

FORM PTO 1390 (REV 11-2000)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER HUBR-1205
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 107049750
INTERNATIONAL APPLICATION NO. PCT/EP00/08088	INTERNATIONAL FILING DATES 18 August 2000	PRIORITY DATE CLAIMED 20 August 1999

ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEOSIDES

APPLICANT(S) FOR DO/EO/US **TISCHER, IHLENFELDT, BARZU, SAKAMOTO, PISTOTNIK, MARLIERE AND POCHET**

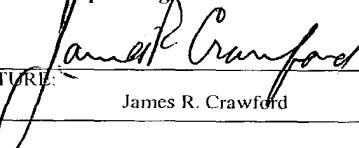
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing 35 U.S.C. 371
3. This is an express request to begin national examination procedures (35 U.S.C. 371 (f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. A copy of the International Application as filed (35 U.S.C. 371 (c)(2))
 - a. is attached hereto (required only if not communicated by the International Bureau).
 - b. has been communicated by the International Bureau.
 - c. is not required, as the application was filed in the United States Receiving Office (RO/US).
6. An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2)).
 - a. is attached hereto.
 - b. has been previously submitted under 35 U.S.C. 154(d)(4).
7. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. are attached hereto (required only if not communicated by the International Bureau).
 - b. have been communicated by the International Bureau.
 - c. have not been made; however, the time limit for making such amendments has NOT expired.
 - d. have not been made and will not be made.
8. An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. A **FIRST** preliminary amendment.
14. A **SECOND** or **SUBSEQUENT** preliminary amendment.
15. A substitute specification.
16. A change of power of attorney and/or address letter.
17. A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. Other items or information: PCT/ISA/210; PCT/IPEA/409

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U.S. APPLICATION NO. (if known, see 37 CFR 1.55)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER	
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17. <input checked="" type="checkbox"/> The following fees are submitted:		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) – (5)):			
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO And International Search Report not prepared by the EPO or JPO \$1040.00			
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ENTER APPROPRIATE BASIC FEE AMOUNT =		\$ 890.00	
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<input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)). \$ 130.00			
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	-20 =	0	X \$
Independent claims	1-3 =	0	X \$
MULTIPLE DEPENDENT CLAIM(s) (if applicable)		X	\$
TOTAL OF ABOVE CALCULATIONS =		\$ 1020.00	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above <input type="checkbox"/> Are reduced by ½.		\$	
SUBTOTAL =		\$ 1020.00	
Processing fee of \$ _____ for furnishing the English translation later than		\$	
<input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). + \$ _____		\$	
TOTAL NATIONAL FEE =		\$ 1020.00	
Fee for recording the enclosed assignment (37 CFR 1.21 (h)). Assignment Must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31) + \$ _____ (per property).		\$	
TOTAL FEES ENCLOSED =		\$ 1020.00	
		Amount to be Refunded:	\$
		Charged:	\$
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>\$1020.00</u> to cover the above fees is enclosed.			
b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.			
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required or credit any overpayment to my Deposit Account No. <u>50-0624</u> . A duplicate copy of this sheet is enclosed.			
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.			
SEND ALL CORRESPONDENCE TO: James R. Crawford FULBRIGHT & JAWORSKI L.L.P. 666 Fifth Avenue New York, New York 10103 (212) 318-3148 Customer No. 24972			
SIGNATURE:  James R. Crawford NAME: _____ 39,155			

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HUBR-1205 (10201511)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Tischer, et al.
Serial No. : 10/049,750
Filed : February 15, 2002
For : ENZYMATIC SYNTHESIS OF
DEOXYRIBONUCLEOSIDES

December 9, 2002

Commissioner of Patents
and Trademarks
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

In advance of prosecution, please amend the above-identified patent application as follows:

IN THE CLAIMS

Cancel claims 3-25, 28-31, 34-36, and 39-41 without prejudice.

REMARKS

This preliminary amendment is the same amendment as that submitted on February 15, 2002, and that amendment also included the statement regarding verification of the computer diskette/sequence listing. Apparently the sequence listing and diskette were indicated as being received, but the amendments to the claims were not entered. An IDS was also submitted on that date, and it is not certain if these were entered. Acknowledgement of receipt of these documents is respectfully requested.

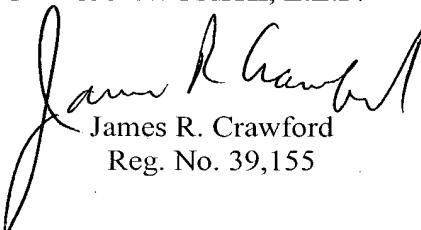
A supplemental preliminary amendment will follow.

It is not believed that any additional claim fees are due in view of this amendment, but any fees that may be due may be charged to deposit account no. 50-0624.

Respectfully submitted,

FULBRIGHT & JAWORSKI, L.L.P.

By



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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEOSIDES

(57) Abstract: The present invention relates to a method for the in vitro enzymatic synthesis of deoxyribonucleosides and enzymes suitable for this method.

Enzymatic synthesis of deoxyribonucleosides

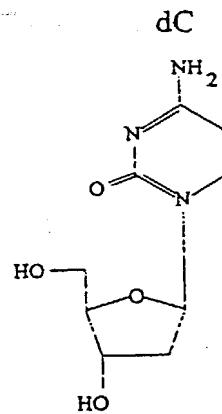
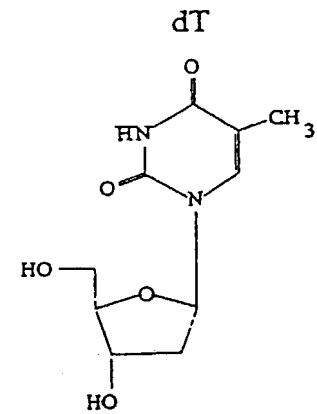
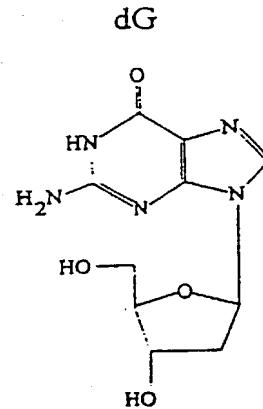
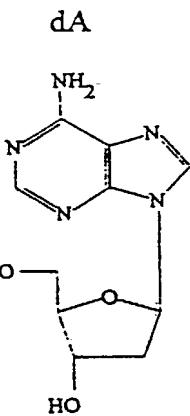
Description

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The present invention relates to a method for the in vitro enzymatic synthesis of deoxyribonucleosides and enzymes suitable for this method.

10 Natural deoxyribonucleosides (deoxyadenosine, dA; deoxyguanosine, dG; deoxycytidine, dC and thymidine, dT) are building blocks of DNA. The N-glycosidic bond between nucleobase and sugar involves the N₁ of a pyrimidine or the N₉ of a purine ring and the C₁ of deoxyribose.

15



25

In the living cells the four deoxyribonucleosides (dN) result from the "salvage pathway" of nucleotide metabolism. A group of enzymes is involved in cellular catabolism of deoxyribonucleosides. Besides deoxyriboaldolase (EC 4.1.2.4) and deoxyribomutase (EC 2.7.5.1), this group also includes thymidine phosphorylase (EC 2.4.2.4) and purine nucleoside phosphorylase (EC 2.4.2.1). These four enzymes are induced by the addition of deoxyribonucleosides to the growth medium. The genes

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coding for these enzymes have been shown to map closely together on the bacterial chromosome (Hammer-Jesperson and Munch-Peterson, Eur.J.Biochem.17 (1970), 397 and literature cited therein). In E.coli the genes as described above are located on the deo operon which exhibits an unusual and complicated pattern of regulation (Valentin-Hansen et al., EMBO J.1 (1982), 317).

Using the enzymes of the deo operon for synthesis of deoxynucleosides was described by C.F.Barbas III (Overproduction and Utilization of Enzymes in Synthetic Organic Chemistry, Ph.D. Thesis (1989), Texas A&M University). He applied phosphopentomutase and thymidine phosphorylase for the synthesis of deoxynucleosides. Deoxyribose 5-phosphate was prepared by chemical synthesis (Barbas III et al., J.Am.Chem.Soc. 112 (1990), 2013-2014), which makes this compound expensive as starting material and not suitable for large scale synthesis. He also made deoxyriboaldolase available as a recombinant enzyme and investigated its synthetic applicability but neither he nor C.-H.Wong (Microbial Aldolases in Carbohydrate Synthesis: ACS Symp.Ser.No.466: Enzymes in Carbohydrate Synthesis, Eds. M.D.Bednarski, E.S.Simon (1991), 23-27) were able to carry out a coupled one-pot synthesis employing all three enzymes. It appears likely that some drawbacks exist which could not be circumvented. Among these drawbacks are insufficient chemical equilibrium, instability of intermediates, such as deoxyribose 1-phosphate and inactivation and inhibition effects of involved compounds on the enzymes.

25

Evidence of an advantageous equilibrium is given by S.Roy et al. (JACS 108 (1986), 1675-78). For the aldolase reaction the equilibrium is on the desired product side (deoxyribose 5-phosphate), for the phosphopentomutase it is on the wrong side (also deoxyribose 5-phosphate) and for the purine nucleoside phosphorylase it is on the desired synthesis product side. The authors suggest coupling of the three enzyme reactions to obtain reasonable yields. Contrary to these suggestions they prepared deuterated

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deoxyguanosine and thymidine in a two step procedure, that is deoxyribose 5-phosphate in a first step and deoxynucleoside in a second step. Isolated yields of the second step were 11% and 5% for deoxyguanosine and thymidine, respectively. These low yields are also obtained in the preparation of arabinose-based nucleosides (Barbas III (1990), *supra*).
5 These low yields indicate serious drawbacks for the use of the enzymes of the deo operon in a synthetic route which have to work in the reverse direction of their biological function, which is degradation of deoxynucleosides.

10

Thus, there does not exist any economical commercial method at present for the enzymatic *in vitro* synthesis of deoxyribonucleosides. Hitherto, for commercial purposes, deoxynucleosides are generated from fish sperm by enzymatic cleavage of DNA. This method, however, involves several
15 disadvantages, particularly regarding difficulties of obtaining the starting material in sufficient quantity and quality.

20 Therefore, it was an object of the invention to provide a method, by means of which the drawbacks of the prior are eliminated at least partially and which allows efficient and economical synthesis of deoxyribonucleosides without any dependence on unreliable natural sources.

25 Surprisingly, it was found that the drawbacks of previous enzymatic synthesis routes can be avoided and deoxyribonucleosides can be obtained in high yields of e.g. at least 80% based on the amount of starting material.

30 In a first aspect, the present invention relates to a method for the *in vitro* enzymatic synthesis of deoxyribonucleosides comprising reacting deoxyribose 1-phosphate (dR1P) and a nucleobase, wherein a deoxyribonucleoside and inorganic phosphate are formed.

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The reaction is catalyzed by an enzyme which is capable of transferring a deoxyribose moiety to a nucleobase, with a deoxyribonucleoside being formed. Preferably, the reaction is catalyzed by a thymidine phosphorylase (TP, EC 2.4.2.4) or a purine nucleoside phosphorylase (PNP, EC 2.4.2.1).

5 For the EC designation of these enzymes and other enzymes mentioned below reference is made to the standard volume Enzyme Nomenclature 1992, Ed. E.C.Webb, Academic Press, Inc.

10 These enzymes and other enzymes mentioned below are obtainable as native proteins from natural sources, i.e. any suitable organisms selected from eukaryotes, prokaryotes and archaea including thermophilic organisms. Further, these enzymes are obtainable as recombinant proteins from any suitable host cell which is transformed or transfected with a DNA encoding said enzyme. The host cell may be a eukaryotic cell, a prokaryotic cell or an archaea cell. Particular preferred sources of native or recombinant TP or PNP are prokaryotic organisms such as E.coli. Recombinant TP may be isolated from E.coli strain pHSP 282 (CNCM I-2186) deposited on April 23, 1999, which is a recombinant E.coli strain transformed with a plasmid containing the E.coli deoA (thymidine phosphorylase) insert. Recombinant 15 PNP may be isolated from E.coli strain pHSP 283 (CNCM I-2187) deposited on April 23, 1999, which is a recombinant E.coli strain transformed with a plasmid containing the E.coli deoD (purine nucleoside phosphorylase) insert. The nucleotide sequence of the TP gene and the corresponding amino acid sequence are shown in SEQ ID NO.1 and 2. The nucleotide sequence of the 20 PNP gene and the corresponding amino acid sequence are shown in SEQ ID NO.15 and 16 and 3 and 4.

25 The nucleobase, to which the deoxyribose unit is transferred, will be selected from any suitable nucleobase. For example, the nucleobase may be a naturally occurring nucleobase such as thymine, uracil, adenine, guanine or hypoxanthine. It should be noted, however, that also non-naturally occurring analogs thereof are suitable as enzyme substrates such

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as 2-thio-uracil, 6-aza-uracil, 5-carboxy-2-thiouracil, 6-aza-thymine, 6-aza-2-thio-thymine and 2,6-diamino-purine.

Preferably the inorganic phosphate is removed from the reaction. This
5 removal is preferably effected by (i) conversion to inorganic pyrophosphate,
(ii) precipitation/complexation and/or (iii) substrate phosphorylation.

Conversion to inorganic pyrophosphate may be effected by a phosphate
transfer from a phosphorylated, preferably polyphosphorylated substrate
10 such as fructose diphosphate (FDP), wherein a phosphate group is cleaved
from the phosphorylated substrate and reacts with the inorganic phosphate,
with inorganic pyrophosphate (PPi) being formed. This phosphate transfer
is preferably catalyzed by a PPi-dependent phosphorylase/kinase, e.g. by a
PPi-dependent phosphofructokinase (PFK-PPi, EC 2.7.1.90), which catalyzes
15 the reaction of fructose diphosphate (FDP) and inorganic phosphate to
fructose 6-phosphate (F6P) and inorganic pyrophosphate. Preferred sources
of PPi-dependent kinases/phosphorylases and genes coding therefor are
from *Propionibacterium freudenreichii* (shermanii) or from potato tubers.

20 Further, the inorganic phosphate may be removed from the reaction by
precipitation and/or complexation which may be effected by adding
polyvalent metal ions, such as calcium or ferric ions capable of precipitating
phosphate or by adding a complex-forming compound capable of
complexing phosphate. It should be noted that also a combination of
25 pyrophosphate formation and complexation/ precipitation may be carried
out.

Furthermore, the removal of inorganic phosphate may be effected by
substrate phosphorylation. Thereby the inorganic phosphate is transferred
30 to a suitable substrate, with a phosphorylated substrate being formed. The
substrate is preferably selected from saccharides, e.g. disaccharides such
as sucrose or maltose. When using disaccharides as substrate, a

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monosaccharide and a phosphorylated monosaccharide are obtained. The phosphate transfer is catalyzed by a suitable phosphorylase/kinase such as sucrose phosphorylase (EC 2.4.1.7) or maltose phosphorylase (EC 2.4.1.8). Preferred sources of these enzymes are *Leuconostoc mesenteroides*,
5 *Pseudomonas saccharophila* (sucrose phosphorylase) and *Lactobacillus brevis* (maltose phosphorylase).

The phosphorylated substrate may be further reacted by additional coupled enzymatic reactions, e.g. into a galactoside (Ichikawa et al., Tetrahedron Lett. 36 (1995), 8731-8732). Further, it should be noted that phosphate removal by substrate phosphorylation may also be coupled with other phosphate removal methods as described above.

Deoxyribose 1-phosphate (dR1P), the starting compound of the method of the invention, is a rather unstable compound, the isolation of which is difficult. In a preferred embodiment of the present invention, d1RP is generated in situ from deoxyribose 5-phosphate (dR5P) which is relatively stable at room temperature and neutral pH. This reaction is catalyzed by a suitable enzyme, e.g. a deoxyribomutase (EC 2.7.5.1) or a phosphopentose 15 mutase (PPM, EC 5.4.2.7) which may be obtained from any suitable source as outlined above. The reaction is preferably carried out in the presence of divalent metal cations, e.g. Mn^{2+} or Co^{2+} as activators. Preferred sources of deoxyribomutase are enterobacteria. Particular preferred sources of native or recombinant PPM are prokaryotic organisms such as *E.coli*.
20 Recombinant PPM may be isolated from *E.coli* strain pHSP 275 (CNCM I-2188) deposited on April 23, 1999, which is a recombinant *E.coli* strain transformed with a plasmid containing the *E.coli* deo B (phosphopentose mutase) insert. The nucleotide sequence of the PPM gene and the corresponding amino acid sequence are shown in SEQ ID NO.17 and 18 and
25 30 5 and 6.

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dR5P may be generated by a condensation of glyceraldehyde 3-phosphate (GAP) with acetaldehyde. This reaction is catalyzed by a suitable enzyme, preferably by a phosphopentose aldolase (PPA, EC 4.1.2.4). The reaction exhibits an equilibrium constant favorable to the formation of the phosphorylated sugar ($K_{eq} = [dR5P]/[acetaldehyde] \times [GAP] = 4.2 \times 10^3 \times M^{-1}$). PPA forms an unstable Schiff base intermediate by interacting with the aldehyde. Particular preferred sources of native or recombinant PPA are prokaryotic organisms such as E.coli. Recombinant PPA may be isolated from E.coli strain pHSP 276 (CNCM I-2189) deposited on April 23, 1999.

This recombinant E.coli strain is transformed with a plasmid containing the deoC (phosphopentosealdolase) insert. The nucleotide sequence of the PPA gene and the corresponding amino acid sequence are shown in SEQ ID NO.19 and 20 and 7 and 8.

GAP is a highly unstable compound and, thus, should be generated in situ from suitable precursors which are preferably selected from fructose 1,6-diphosphate (FDP), dihydroxyacetone (DHA) and/or glycerolphosphate (GP), with FDP being preferred.

FDP can be converted by an FDP aldolase (EC 4.1.2.13) selected from FDP aldolases I and FDP aldolases II to GAP and dihydroxyacetone phosphate ($K_{eq} = [FDP]/[GAP] \times [DHAP] = 10^4 M^{-1}$). The two families of FDP aldolases giving identical end products (GAP and DHAP) via two chemically distinct pathways may be used for this reaction. FDP aldolase I forms Schiff base intermediates like PPA, and FDP aldolase II which uses metals (Zn^{2+}) covalently bound to the active sites to generate the end products. FDP-aldolase I is characteristic to eukaryotes, although it is found in various bacteria. FDP-aldolase II is more frequently encountered in prokaryotic organisms. If FDP-aldolase reacts with FDP in the presence of acetaldehyde, the latter compound can interact with DHAP to yield an undesired condensation by-product named deoxyxylolose 1-phosphate (dX1P). Thus,

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the reaction is preferably conducted in a manner by which the generation of undesired side products is reduced or completely suppressed.

Particular preferred sources of native or recombinant FDP aldolases are 5 prokaryotic or eukaryotic organisms. For example, FDP aldolase may be isolated from rabbit muscle. Further, FDP aldolase may be obtained from bacteria such as E.coli. Recombinant FDP aldolase may be isolated from recombinant E.coli strain pHSP 284 (CNCM I-2190) which is transformed with a plasmid containing the E.coli fba (fructose diphosphate aldolase) 10 insert. The nucleotide sequence of the E.coli FDP aldolase gene and the corresponding amino acid sequence are shown in SEQ ID NO.9 and 10.

On the other hand, GAP may be generated from DHAP and ATP, with 15 dihydroxyacetone phosphate (DHAP) and ADP being formed and subsequent isomerization of DHAP to GAP in a reaction catalyzed by a glycerokinase (GK, EC 2.7.1.30) and a triose phosphate isomerase (TIM, EC 5.3.1.1). Suitable glycerokinases are obtainable from E.coli, suitable triose phosphate isomerases are obtainable from bovine or porcine muscle.

20 In a still further embodiment of the present invention GAP may be generated from glycerol phosphate (GP) and O₂, with DHAP and H₂O₂ being formed and subsequent isomerization of DHAP to GAP in a reaction catalyzed by a glycerophosphate oxidase (GPO, EC 1.1.3.21) and a triose phosphate isomerase (TIM, EC 5.3.1.1). Suitable glycerophosphate oxidases are 25 obtainable from Aerococcus viridans.

30 In an alternative embodiment of the present invention deoxyribose 5-phosphate (dR5P) is generated by phosphorylation of deoxyribose. Preferably this reaction is carried out in the presence of a suitable enzyme, e.g. a deoxyribokinase (dRK, EC 2.7.1.5) which may be obtained from prokaryotic organisms, particularly *Salmonella typhi* and in the presence of

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ATP. The nucleotide sequence of the *Salmonella* dRK gene and the corresponding amino acid sequence are shown in SEQ ID NO.11 and 12.

By the reaction as outlined above deoxyribonucleosides are obtained which contain a nucleobase which is accepted by the enzymes TP and/or PNP. TP is specific for thymidine (T), uracil (U) and other related pyrimidine compounds. PNP uses adenine, guanine, hypoxanthine or other purine analogs as substrates.

5 The synthesis of deoxyribonucleosides which are not obtainable by direct condensation such as deoxycytosine (dC), thus, require an additional enzymatic reaction, wherein a deoxyribonucleoside containing a first nucleobase is reacted with a second nucleobase, with a second ribonucleoside containing the second nucleobase being formed. The second nucleobase is preferably selected from cytosine and analogs thereof such as 5-azacytosine. It should be noted, however, that also other nucleobases such as 6-methyl purine, 2-amino-6-methylmercaptopurine, 6-dimethylaminopurine, 2,6-dichloropurine, 6-chloroguanine, 6-chloropurine, 6-azathymine, 5-fluorouracil, ethyl-4-amino-5-imidazole carboxylate, 20 imidazole-4-carboxamide and 1,2,4-triazole-3-carboxamide may be converted to the corresponding deoxyribonucleoside by this nucleobase exchange reaction (Beaussire and Pochet, Nucleosides & Nucleotides 14 (1995), 805-808, Pochet et al., Bioorg.Med.Chem.Lett.5 (1995), 1679-1684, Pochet and Dugué, Nucleosides & Nucleotides 17 (1998), 2003-25 2009, Pistotnik et al., Anal.Biochem.271 (1999), 192-199). This reaction is preferably catalyzed by an enzyme called nucleoside 2-deoxyribosyltransferase (NdT, EC 2.4.2.6) which transfers the glycosyl moiety from a first deoxynucleoside to a second nucleobase, e.g. cytosine. A preferred source of native or recombinant NdT are prokaryotic organisms 30 such as lactobacilli, particularly *Lactobacillus leichmannii*. Recombinant NdT may be isolated from recombinant *E.coli* strain pHSP 292 (CNCM I-2191) deposited on April 23, 1999, which is transformed with a plasmid

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containing the *L.leichmannii* NdT (nucleoside 2-deoxyribosyltransferase) insert. The nucleotide sequence of the NdT gene and the corresponding amino acid sequence are shown in SEQ ID NO.13 and 14.

5 A further aspect of the present invention is a method for the in vitro enzymatic synthesis of deoxyribonucleosides comprising the steps of: (i) condensing glyceraldehyde 3-phosphate (GAP) with acetaldehyde to deoxyribose 5-phosphate (dR5P), (ii) isomerizing deoxyribose 5-phosphate to deoxyribose 1-phosphate (dR1P) and (iii) reacting deoxyribose 1-phosphate and a nucleobase, wherein a deoxyribonucleoside and inorganic phosphate are formed. Preferably, the reaction is carried out without isolating intermediate products and, more preferably, as a one-pot reaction. Further, the removal of the inorganic phosphate from the reaction is preferred.

15

As outlined above, the glyceraldehyde 3-phosphate may be generated from FDP, DHA and/or GP. Preferably, FDP is used as a starting material.

20 In order to avoid the production of undesired by-products and the toxic effects of acetaldehyde, the course of the reaction is preferably controlled by suitable means. Thus, preferably, the reaction is carried out in a manner such that the acetaldehyde concentration in step (ii) is comparatively low, e.g. less than 100 mM, particularly less than 50 mM, e.g. by adding the acetaldehyde in portions or continuously during the course of the reaction and/or by removing excess acetaldehyde. Further, it is preferred that before step (ii) excess starting materials and/or by-products, particularly fructose 25 1,6-diphosphate and/or deoxyxylulose 1-phosphate (dX1P), are removed. This removal may be effected by chemical and/or enzymatic methods, e.g. precipitating FDP with ferric salts or enzymatically degrading X1P via dihydroxyacetone phosphate. Alternatively or additionally the reaction 30 conditions may be adjusted such that before step (ii) no substantial amounts, preferably less than 10 mM, of starting materials and/or by-

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products, particularly fructose 1,6-diphosphate and/or deoxyxylulose 1-phosphate, are present in the reaction mixture.

In still another embodiment, the present invention relates to a method for the in vitro enzymatic synthesis of deoxyribonucleosides comprising the steps of: (i) phosphorylating deoxyribose to deoxyribose 5-phosphate, (ii) isomerizing deoxyribose 5-phosphate to deoxyribose 1-phosphate and (iii) reacting deoxyribose 1-phosphate and nucleobase, wherein a deoxyribonucleoside and inorganic phosphate are formed. Preferably, these reactions are carried out with isolating intermediate products and, more preferably, as a one-pot reaction. To obtain a better yield the removal of inorganic phosphate from step (iii) is preferred.

By the process as described above naturally occurring deoxyribonucleosides such as dA, dG, dT, dU and dT but also analogs thereof containing non-naturally occurring nucleobases and/or non-naturally occurring deoxyribose sugars such as 2'-deoxy-3'-azido-deoxyribose or 2'-deoxy-4'-thio-deoxyribose may be produced.

The deoxyribonucleosides obtained may be converted to further products according to known methods. These further reaction steps may comprise the synthesis of deoxyribonucleoside mono-, di- or triphosphates, of H-phosphonates or phosphoramidites. Additionally or alternatively, labelling groups such as radioactive or chemical labelling groups may be introduced into the deoxyribonucleosides.

Still a further aspect of the present invention is the use of an isolated nucleic acid molecule encoding a nucleoside 2-deoxyribosyl transferase (NdT, EC 2.4.2.6) for the preparation of an enzyme in an in vitro enzymatic synthesis process, wherein a deoxyribonucleoside containing a first nucleobase is reacted with a second nucleobase, with a deoxyribonucleoside containing the second nucleobase being formed. The

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second nucleobase is preferably selected from cytidine and analogs thereof, 2,6-dichloro-purine, 6-chloro-guanine, 6-chloro-purine, 6-aza-thymine and 5-fluoro-uracil. The first nucleobase is preferably selected from thymine, guanine, adenine or uracil.

5

More preferably, the nucleic acid molecule encoding an NdT comprises (a) the nucleotide sequence shown in SEQ ID NO.13 or its complementary sequence, (b) a nucleotide sequence corresponding to the sequence of (a) in the scope of degeneracy of the genetic code or (c) the nucleotide sequence hybridizing under stringent conditions to the sequence (a) and/or (b). Apart from the sequence of SEQ ID NO.13 the present invention also covers nucleotide sequences coding for the same polypeptide, i.e. they correspond to the sequence within the scope of degeneracy of the genetic code, and nucleotide sequence hybridizing with one of the above-mentioned sequences under stringent conditions. These nucleotide sequences are obtainable from SEQ ID NO.13 by recombinant DNA and mutagenesis techniques or from natural sources, e.g. from other *Lactobacillus* strains.

Stringent hybridization conditions in the sense of the present invention are defined as those described by Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press (1989), 1.101-1.104. According to this, hybridization under stringent conditions means that a positive hybridization signal is still observed after washing for one hour with 1 x SSC buffer and 0.1% SDS at 55°C, preferably at 62°C and most preferred at 68°C, in particular, for one hour in 0.2 x SSC buffer and 0.1% SDS at 55°C, preferably at 62°C and most preferred at 68°C.

Moreover, the present invention also covers nucleotide sequences which, on nucleotide level, have an identity of at least 70%, particularly preferred at least 80% and most preferred at least 90% to the nucleotide sequence shown in SEQ ID NO.13. Percent identity are determined according to the following equation:

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$$I = \frac{n}{L} \times 100$$

5

wherein I are percent identity, L is the length of the basic sequence and n is the number of nucleotide or amino acid difference of a sequence to the basic sequence.

10 Still another subject matter of the present invention is a recombinant vector comprising at least one copy of the nucleic acid molecule as defined above, operatively linked with an expression control sequence. The vector may be any prokaryotic or eukaryotic vector. Examples of prokaryotic vectors are chromosomal vectors such as bacteriophages (e.g. bacteriophage Lambda) and extrachromosomal vectors such as plasmids (see, for example, Sambrook et al., *supra*, Chapter 1-4). The vector may also be a eukaryotic vector, e.g. a yeast vector or a vector suitable for higher cells, e.g. a plasmid vector, viral vector or plant vector. Suitable eukaryotic vectors are described, for example, by Sambrook et al., *supra*, Chapter 16. The invention moreover relates to a recombinant cell transformed with the nucleic acid or the recombinant vector as described above. The cell may be any cell, e.g. a prokaryotic or eukaryotic cell. Prokaryotic cells, in particular, *E.coli* cells, are especially preferred.

15

20

25 The invention refers to an isolated polypeptide having NdT activity encoded by the above-described nucleic acid and its use for the preparation of deoxyribonucleosides. Preferably, the polypeptide has the amino acid sequence shown in SEQ ID NO.14 or an amino acid sequence which is at least 70%, particularly preferred at least 80% and most preferred at least 90% identical thereto, wherein the identity may be determined as described above.

30

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Finally, the present invention also relates to the use of isolated nucleic acid molecules having thymidine phosphorylase (TP), purine nucleoside phosphorylase (PNP), phosphopentose mutase (PPM), phosphopentose aldolase (PPA), FDP aldolase and deoxyribokinase (dRK) activity for the preparation of an enzyme for a method for the in vitro synthesis of deoxynucleosides. Preferably, these nucleic acids are selected (a) from a nucleotide sequence shown in SEQ ID NO.1, 3, 5, 7, 9 or 11 or their complementary sequences; (b) a nucleotide sequence corresponding to a sequence of (a) within the scope of degeneracy of the genetic code or (c) a nucleotide sequence hybridizing under stringent conditions to a sequence (a) and/or (b).

Isolated polypeptides having TP, PNP, PPM, PPA, FDP aldolase or dRK activity encoded by the above-described nucleic acids may be used for the preparation of deoxyribonucleosides. Preferably, these polypeptides have the amino acid sequence shown in SEQ ID NO.2, 4, 16, 6, 18, 8, 20, 10 or 12 or an amino acid sequence which is at least 70%, particularly preferred at least 80% and most preferred at least 90% identical thereto, wherein the identity may be determined as described above.

An isolated nucleic acid molecule encoding a dRK may be used for the preparation of an enzyme for an in vitro method for the enzymatic synthesis of deoxyribonucleosides comprising the step of phosphorylating deoxyribose to deoxyribose 5-phosphate, wherein said nucleic acid molecule comprises (a) the nucleotide sequence shown in SEQ ID NO.11 or its complementary sequence, (b) a nucleotide sequence corresponding to the sequence of (a) in the scope of the degeneracy of the genetic code or (c) a nucleotide sequence hybridizing under stringent conditions to the sequence of (a) and/or (b). Correspondingly, an isolated polypeptide having dRK activity is suitable for an in vitro method for the enzymatic synthesis of deoxyribonucleosides as outlined above.

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The E.coli strains pHSP 282 (CNCM I-2186), pHSP 283 (CNCM I-2187), pHSP 275 (CNCM I-2188), pHSP 276 (CNCM 2189), pHSP 284 (CNCM I-2190) and pHSP 292 (CNCM I-2191) were deposited according to the regulations of the Budapest Treaty on April 23, 1999 at the Collection Nationale de Culture de Microorganismes, Institut Pasteur, 25, Rue de Docteur Roux, 75724 Paris Cedex 15.

Description of figures

10 **Figure 1** shows the synthesis of dR5P according to Example 12.

Figure 2 shows the synthesis of deoxyadenosine according to Example 12.

15 **Figure 3** shows the synthesis of deoxyadenosine according to Example 13.

Figure 4 shows the synthesis of dG-NH₂ according to Example 14.

20 **Example 1**

Sources of Enzymes

L-glycerol 3-phosphate oxidase (1.1.3.21) from *Aerococcus viridans*, sucrose phosphorylase (2.4.1.7), fructose 6-phosphate kinase (2.7.1.90) 25 from *Propionibacterium freudenreichii*, rabbit muscle aldolase (RAMA), formate dehydrogenase, glycerolphosphate dehydrogenase (GDH), triosephosphate isomerase (TIM), catalase, glycerol 3-phosphate oxidase and maltose phosphorylase were obtained from commercial sources (Roche Diagnostics, Sigma) or as described in the literature.

30

FDP aldolase II (4.1.2.13), phosphopentose aldolase (PPA, EC 4.1.2.4), phosphopentose mutase (PPM, EC 5.4.2.7), thymidine phosphorylase (TP,

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EC 2.4.2.4), purine nucleoside phosphorylase (PNP, EC 2.4.2.1), nucleoside 2-deoxyribosyl transferase (NdT, EC 2.4.2.6) were obtained from *E.coli* strains deposited at CNCM (see above).

5 **Example 2**

Protocol of the synthesis of deoxyadenosine

Reaction mixture A was prepared by adding acetaldehyde (final 10 concentration 250 mM), FDP aldolase II (0.5 U/ml), PPA (2.5 U/ml) to 20 ml of 100 mM fructose-1,6-diphosphate (FDP), pH 7.6 and incubating overnight at 4°C.

Mixture B was prepared by adding MnCl₂ (final concentration 0.6 mM), 15 glucose 1,6-diphosphate (15 µM), PPM (1.5 U/ml), PNP (0.4 U/ml), SP (1.5 U/ml) pentosephosphate aldolase, PPA (2 U/ml) and FDP aldolase II (0.5 U/ml) to 10 ml 0.9 M sucrose, pH 7.6, at room temperature.

2 ml of A were added over B at a temperature of 20°C. After 1 hour 2.5 ml 20 A were added. After another hour 3.0 ml A were added. After another 1.5 h 3.5 ml A were added. After another 1.5 h 4 ml A were added and after another 1-1.5 h 5 ml A were added and left to stand overnight.

At each time of addition of A the amounts of FDP, dR5P, dX1P and dA in 25 the reaction mixture were determined and the yield was calculated. The concentration of acetaldehyde was kept between 20-30 mM. The results are shown in Table 1:

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Table 1

Time (h)	Volume (ml)	Concentrations (mM)			Yield (mmol) dA
		dR5P	dA	dX1P	
0	12	4	0	1.2	0
1	12	3.4	3.2	1	0.04
2	14.5	7.9	8.0	2.6	0.12
3.5	17.5	13	16.2	4.3	0.28
5	21	11.7	21.7		0.46
6	25		23.7		0.59
22	30	11	40.4	13.2	1.21
30	30		50.3		1.51
54	30	8.9	60.6		1.82

15 The starting amount of FDP was 1.92 mmol. The amount after completion of reaction was 0.150 mmol. Thus, 1.77 mmol were consumed, theoretically corresponding to 3.54 mmol equivalents dA. The amount of dA formed was 1.82 mmol, leading to a yield of 51.4% based on the amount of FDP.

20

Example 3

Removal of excess FDP by means of FeCl_3

25 1.4 g (2.55 mmol) trisodium-fructose-1,6-disphosphate-octahydrate and 430 μl (335 mg, 7.6 mmol) acetaldehyde were dissolved in 15 ml of water at 4°C. A pH of 7.9 was adjusted by means of sodium hydroxide solution. 150 U pentosephosphate aldolase (PPA) were added, and cold water (4°C)

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was added to give 20 ml. After addition of 50 U E.coli aldolase II the mixture was stored at 4°C. After 2 h another 75 U PPA and 50 µl acetaldehyde (390 mg, 8.9 mmol) were added. After 20 h 500 U triosephosphate isomerase (TIM) were added. After 120 h the solution contained about 68 mM FDP, about 12 mM dX1P and about 45 mM dR5P. The reaction was stopped by adding 900 µl of a 2 M solution of iron(III) chloride in 0.01 M hydrochloric acid. The precipitate was centrifuged and washed, the resulting solution contained about 4 mM dX1P, about 9 mM FDP and about 25 mM dR5P.

10

Example 4

Removal of excess FDP and dX1P by degradation via DHAP

15 576 mg (1.05 mmol) trisodium-fructose-1,6-disphosphate-octahydrate were dissolved in 8 ml water, and the pH was adjusted at 8.1 by means of sodium hydroxide solution. 75 U PPA and 27 U rabbit muscle aldolase (RAMA) were added, and water was added to give 10 ml. 570 µl (440 mg, 10 mmol) acetaldehyde were added. The reaction was stored at 4°C. After 20 100 h the solution contained about 110 mM dX1P, about 5 mM FDP and about 85 mM dR5P (about 870 µmol). The reaction was stopped by adding hydrochloric acid until a pH of 2 was reached. After adding sodium hydroxide solution to give a pH of 5.5 the solution was stored.

25 For removing dX1P the acetaldehyde was evaporated and the solution was diluted with water to reach 30 ml. It was mixed with 3 ml 2.65 M sodium formate solution (8 mmol), and sodium hydroxide solution was added until a pH of 7.4 was reached. 23 U formate dehydrogenase (FDH), 6 mg NADH, 16 U RAMA and 20 U glycerolphosphate dehydrogenase (GDH) were 30 added.

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After 24 h at room temperature the concentrations of dX1P and FDP are below 3 mM, the loss of dR5P is less than 10%.

Example 5

5

Preparation of dR5P via G3P

1.1 g (2.0 mmol) trisodium-fructose-1,6-disphosphate-octahydrate were dissolved in 8 ml water. 1.58 mol of a 2.65 M sodium formate solution (4.2 mmol) and 14.2 mg NADH were added. A pH of 7.0 was adjusted by means of NaOH. After addition of 36 U RAMA, 50 U triosephosphate isomerase (TIM), 34 U GDH and 35 U FDH water was added to give 12 ml.

10 After incubation of 40 h at room temperature the FDP content was below 3 mM. The enzymes were denatured by acidification with hydrochloric acid to reach a pH of 2. Subsequently, the pH of the solution was adjusted at 4 and the solids were centrifuged and filtered off, respectively. Through dilution during purification a total volume of 25 ml was reached which contained about 160 mM of glycerol-3-phosphate (G3P).

15

20 4 ml of this solution (about 640 μ mol G3P) were adjusted at a pH of 7.8 by means of sodium hydroxide solution. 7.8 kU catalase, 500 U TIM and 13 U glycerol 3-phosphate oxidase are added. The mixture was stirred very slowly in an open flask. After 30 min 18 U PPA were added. Acetaldehyde was added in portions of 30 μ l (23.5 mg, 530 μ mol) after 30, 60, 120, 180 and 240 min. After 24 h another 15 U PPA, 2.5 kU TIM and 100 μ l (78 mg, 1.8 mmol) acetaldehyde were added. After 30 h the batch is sealed after addition of another 100 μ l acetaldehyde. After a total of 45 h a concentration of about 60 mM dR5P was achieved and the reaction is completed. For preparing 2'-deoxyadenosine (e.g. Example 7) excess acetaldehyde must be distilled off.

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Example 6

Preparation of a dR5P solution containing small amounts of dX1P or FDP

5 A solution of 60 mmol/l FDP and 120 mmol/l acetaldehyde having pH 7.4
was kept at a temperature of 15°C. 5 ml thereof were mixed with 4 U
aldolase II, 2 U TIM and 40 U PPA and kept at 15°C. After 4, 8.5, 16.5
and 24 h 12 U PPA and 100 µl of a 34 vol.-% solution of acetaldehyde in
water (26.4 mg, 600 µmol) were added each. After 40 h the solution was
10 allowed to reach room temperature. After 90 h the reaction solution had
reached concentrations of about 3 mM FDP, about 4 mM dX1P and at least
70 mM dR5P. For stopping the reaction and removing acetaldehyde about
20% of the volume were distilled off.

15 **Example 7**

Preparation of deoxyadenosine (dA) from dR5P by means of barium acetate

dR5P was used in the form of a solution prepared according to Examples 3-
20 6. For instance, 10 ml of a solution of Example 6 diluted to have 70 mM
dR5P (700 µmol dR5P) were mixed with 40 mg (300 µmol) adenine, 41 µg
(50 nmol) tetracyclohexylammonium-glucose-1,6-disphosphate, 396 µg (2
µmol) manganese-II-acetate-tetrahydrate, 10 U pentosephosphate mutase
(PPM) and 30 U purine-nucleoside phosphorylase (PNP). After 3 h another
25 27 mg (200 µmol) adenine and 26 mg (100 µmol) barium acetate were
added.

A further amount of 26 mg barium acetate was added after 4 h, one of 40
30 mg adenine after 7 h. After 10 h the reaction was completed. The solution
had a concentration of 45 mM dA.

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Example 8

Preparation of deoxyadenosine (dA) from dR5P by means of sucrose phosphorylase

5

10 ml of a solution of Example 6 diluted to 55 mM dR5P (550 μ mol dR5P) were mixed with 81 mg (600 μ mol) adenine, 41 μ g (50 nmol) tetracyclohexylammonium-glucose-1,6-disphosphate, 396 μ g (2 μ mol) manganese-II-acetate-tetrahydrate, 10 U pentosephosphate mutase (PPM) 10 15 U purine nucleoside phosphorylase (PNP), 25 U sucrose phosphorylase and 340 mg (1 mmol) cane sugar.

10

After 3 h at room temperature the reaction was completed. The solution had a concentration of about 50 mM dA.

15

Example 9

Preparation of deoxyadenosine (dA) from dR5P by means of maltose phosphorylase

20

10 ml of a solution of dR5P diluted to 55 mM were mixed at pH 7.0 with 81 mg (600 μ mol) adenine, 41 μ g (50 nmol) glucose 1,6-diphosphate, 396 μ g (2 μ moles) manganese II-acetate tetrahydrate, 5 units pentose phosphate mutase (PPM), 10 units purine nucleoside phosphorylase, (PNP), 25 20 units maltose phosphorylase and 1080 mg (3 mmol) maltose.

25

After 12h at room temperature the reaction was completed. The solution had a concentration of 49 mM dA.

30

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Example 10

Preparation of deoxycytosine (dC) from dR5P by means of sucrose phosphorylase

5

20 ml of a solution of dR5P diluted to 70 mM were mixed at pH 7.0 with 5.4 mg adenine (0.04 mmoles), 155 mg cytosine (1.4 mmoles), 82 μ g (100 nmoles) glucose 1,6-diphosphate, 792 μ g (4 μ moles) manganese II-acetate-tetrahydrate, 20 units PPM, 30 units PNP, 50 units 2-deoxyribosyl transferase (NdT), 50 units sucrose phosphorylase and 2.05 g (6 mmoles) sucrose.

10 After 18h at 30°C the solution had a concentration of 62 mM dC.

15

Example 11

Preparation of deoxyguanosine (dG) from dR5P by means of sucrose phosphorylase

20

20 ml of a solution of dR5P diluted to 70 mM were mixed at pH 7.0 with 91 mg guanine (0.6 mmoles), 82 μ g (100 nmoles) glucose 1,6-diphosphate, 792 μ g (4 μ moles) manganese II-acetate-tetrahydrate, 20 units PPM, 10 units PNP, 20 units sucrose phosphorylase and 2.05 g (6 mmoles) sucrose.

25

After 18h at 37°C the dG formed corresponds to 0.5 mmoles.

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Example 12

Two step procedure of dA synthesis

5 In the first step dR5P was prepared by adding FDP-Aldolase II (AldII) from E. coli, pentosephosphate aldolase (PPA) from E. coli and triosephosphate isomerase (TIM) from E. coli to fructose-1,6-bisphosphate (FDP) and acetaldehyde (AcAld) essentially according to Ex. 6. FDP trisodium salt was mixed in a final concentration of 75 mM with AcAld (100 mM final concentration). The pH was adjusted to 7,4 by addition of sodium hydroxide. The reaction was started by adding PPA (12 kU/l), Ald II (0,3 kU/l) and TIM (2,5 kU/l). At 4 h 117 mM AcAld, at 7 h 117 mM AcAld, PPA 6 kU/l, TIM 2,5 kU/l and at 12 h 117 mM AcAld were added. The reaction was run at 21 °C. Conversion was monitored by enzymatical assay using 10 step by step glycerol-3-phosphate dehydrogenase (GDH), rabbit muscle aldolase (RAMA), triosephosphate isomerase (TIM), pentosephosphate aldolase (PPA) in the presence of NADH (0,26 mM in 300 mM triethanol 15 amine buffer pH 7.6). Conversion is shown in Fig. 1.

15 After yielding approx. 95 mM dR5P the enzymes were deactivated by heating to 65 °C for 10 min. and excess of AcAld was removed by evaporation. In the second step dR5P in a final concentration of 64 mM was converted to deoxyadenosine (dA) by adding adenine (A, final concentration 20 58 mM) in the presence of 300 µM MnCl₂, 5 µM Glucose-1,6-bisphosphate, 25 pentosephosphate mutase from E. coli (PPM, 2 kU/l), purine nucleoside phosphorylase from E. coli (PNP, 1 kU/l). The synthesis was run at 20 °C, pH 7.4. In one experiment 200 mM sucrose and 0.6 kU/l sucrose phosphorylase (SP) from Leuconostoc mes. were added at t=2 h (see arrow in Fig. 2, rhombus, solid line), in a second experiment addition of SP 30 was omitted (squares, dotted line). The conversion was monitored by RP-HPLC (column Hypersil ODS 5 µm, 250 x 4,6 mm; eluent: 30 mM potassium phosphate, 5 mM tetrabutyl ammoniumhydrogensulfate pH 6.0/

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1 % acetonitrile, flow rate: 1 ml/min, column temp.: 35 °C, det.: UV at 260 nm) and is shown in Fig. 2.

Example 13

5 dR5P was prepared by adding FDP-Aldolase II (AldII) from *E. coli*, pentosephosphate aldolase (PPA) from *E. coli* and trisosephosphate isomerase (TIM) from *E. coli* to fructose-1,6-bisphosphate (FDP) and acetaldehyde (AcAld) essentially according to Ex. 6. Excess of AcAld was
10 removed by evaporation. dR5P in a final concentration of 60 mM was converted to deoxyadenosine (dA) by adding adenine (A, final concentration 58 mM) in the presence of 300 μ M MnCl₂, 5 μ M Glucose-1,6-bisphosphate, pentosephosphate mutase from *E. coli* (PPM, 1,5 kU/l), purine nucleoside phosphorylase from *E. coli* (PNP, 1 kU/l). The synthesis was run at 20 °C,
15 pH 7.4. After 24 h sucrose in a final concentration of 200 mM and sucrose phosphorlyase from *Leuconostoc mes.* (1 kU/l) were added. Conversion was monitored by RP-HPLC (dA, A, see ex. 12)) resp. enzymatical assay (dR5P, using step by step glycerol-3-phosphate dehydrogenase (GDH), rabbit muscle aldolase (RAMA), trisosephosphate isomerase (TIM),
20 pentosephosphate aldolase (PPA) in the presence of NADH (0,26 mM in 300 mM Triethanol amine buffer pH 7.6)) and phosphomolybdate complexing of inorg. phosphate (Sigma, Proc. No. 360-UV). This is shown in Fig. 3.

25 **Example 14**

dR5P was essentially prepared according according to Ex. 6. dR5P in a final concentration of 80 mM was then converted to deoxy-6-aminoguanosine (dG-NH₂) by adding 2,6-Diaminopurine (DAP, final concentration 77 mM) in
30 the presence of 200 mM sucrose, 300 μ M MnCl₂, 5 μ M Glucose-1,6-bisphosphate, pentosephosphate mutase from *E. coli* (PPM, 2,5 kU/l), purine nucleoside phosphorylase from *E. coli* (PNP, 1 kU/l), sucrose

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phosphorylase from Leuconostoc mes. (SP, 1,5 kU/l) . The synthesis was run at 20°C pH 7.4. After 2,5h, 5 h and 20,5 h additional amounts of enzymes were added: 2,5 h PPM (2,5 kU/l), PNP (1 kU/l, SP (1,5 kU/l), 5 h PPM (2,5 kU/l), SP (1,5 kU/l), 20,5 h: PPM (2,5 kU/l), SP (1,5 kU/l). The conversion was monitored by RP-HPLC (column Hypersil ODS 5 µm, 250 x 4,6 mm; eluent: 30 mM potassium phosphate, 5 mM tetrabutyl ammoniumhydrogensulfate pH 6.0/ 1 % acetonitrile, flow rate: 1 ml/min, column temp.: 35°C, det.: UV at 216 nm) and is shown in Fig. 4.

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New Claims

1. A method for the in vitro enzymatic synthesis of deoxyribonucleosides comprising reacting deoxyribose 1-phosphate (dR1P) and a nucleobase, wherein a deoxyribonucleoside and inorganic phosphate are formed and wherein the inorganic phosphate is removed.
2. The method of claim 1, wherein the reaction is catalyzed by a thymidine phosphorylase (TP, EC 2.4.2.4) or a purine nucleoside phosphorylase (PNP, EC 2.4.2.1).
3. The method of any one of the previous claims, wherein the nucleobase is selected from the group consisting of thymine, uracil, adenine, guanine and hypoxanthine and analogs thereof, e.g. 2-thio-uracil, 6-aza-uracil, 5-carboxy-2-thio-uracil, 6-aza-thymine, 6-aza-2-thio-thymine and 2,6-diamino-purine.
4. The method of any one of the previous claims, wherein the removal of the inorganic phosphate is effected by (i) conversion to inorganic pyrophosphate, (ii) precipitation, (iii) complexation and/or (iv) substrate phosphorylation.
5. The method of claim 4, wherein the inorganic phosphate is converted to pyrophosphate by a phosphate transfer from fructose-diphosphate (FDP) under formation of fructose-6-phosphate (F6P).
6. The method of claim 5, wherein the phosphate transfer is catalyzed by a PPi-dependent phosphofructokinase (PFK-PPi, EC 2.7.1.90).

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7. The method of claim 5 or 6, wherein the inorganic pyrophosphate is removed by precipitation.
8. The method of claim 4, wherein the inorganic phosphate is transferred to a disaccharide, particularly sucrose or maltose under formation of a monosaccharide and a phosphorylated monosaccharide.
9. The method of claim 8, wherein the phosphate transfer is catalyzed by a sucrose phosphorylase (EC 2.4.1.7) or a maltose phosphorylase (EC 2.4.1.8).
10. The method of claim 9, wherein the phosphorylated monosaccharide is further reacted.
11. The method of any one of the previous claims, wherein the deoxyribose-1-phosphate is generated from deoxyribose 5-phosphate (dR5P).
12. The method of claim 11, wherein the reaction is catalyzed by a deoxyribomutase (EC 2.7.5.1) or a phosphopentose mutase (PPM, EC 5.4.2.7).
13. The method of claim 11 or 12, wherein the deoxyribose-5-phosphate is generated by a condensation of glyceraldehyde 3-phosphate (GAP) with acetaldehyde.
14. The method of claim 13, wherein the reaction is catalyzed by a phosphopentose aldolase (PPA, EC 4.1.2.4).
15. The method of claim 13 or 14, wherein the glyceraldehyde 3-phosphate is generated from fructose 1,6-diphosphate,

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dihydroxyacetone (DHA) and/or glycerolphosphate.

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16. The method of claim 15, wherein the glyceraldehyde 3-phosphate is generated from fructose 1,6-diphosphate in a reaction catalyzed by an FDP-alcoholase (EC 4.1.2.13) selected from FDP-alcoholases I and FDP-alcoholases II.
17. The method of claim 15, wherein the glyceraldehyde 3-phosphate is generated from dihydroxyacetone and ATP under formation of dihydroxyacetone phosphate (DHAP) and ADP and subsequent isomerization of DHAP to GAP in a reaction catalyzed by a glycerokinase (GK, EC 2.7.1.30) and a triose phosphate isomerase (TIM, EC 5.3.1.1).
18. The method of claim 15, wherein the glyceraldehyde 3-phosphate is generated from glycerol phosphate (GP) and O₂ under formation of dihydroxyacetone phosphate (DHAP) and H₂O₂ and subsequent isomerization of DHAP to GAP in a reaction catalyzed by a glycerophosphate oxidase (GPO, EC 1.1.3.21) and a triose phosphate isomerase (TIM, EC 5.3.1.1).
19. The method of claim 11 or 12, wherein the deoxyribose 5-phosphate is generated by a phosphorylation of deoxyribose.
20. The method of claim 19, wherein the reaction is catalyzed by a deoxyribokinase (dRK, EC 2.7.1.15).
21. The method of claim 20, wherein a dRK obtainable from *Salmonella typhi* is used which is encoded by (a) the nucleotide sequence shown in SEQ ID NO.11 or its complementary sequence, (b) a nucleotide sequence corresponding to the sequence of (a) in the scope of the degeneracy of the genetic code or (c) a nucleotide sequence

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hybridizing under stringent conditions to the sequence of (a) and/or (b).

22. The method of any one of the previous claims, wherein a deoxyribonucleoside containing a first nucleobase is further reacted with a second nucleobase under formation of a deoxyribonucleoside containing the second nucleobase.

23. The method of claim 22, wherein said second nucleobase is selected from cytidine and analogs thereof, e.g. 5-aza-cytidine, 2,6-dichloropurine, 6-chloro-guanine, 6-chloro-purine, 6-aza-thymine and 5-fluorouracil.

24. The method of claim 23, wherein the reaction is catalyzed by a nucleoside 2-deoxyribosyl transferase (NdT, EC 2.4.2.6).

25. The method of claim 24, wherein an NdT obtainable from *Lactobacillus leichmannii* is used which is encoded by (a) the nucleotide sequence shown in SEQ ID NO.13 or its complementary sequence, (b) a nucleotide sequence corresponding to the sequence of (a) in the scope of the degeneracy of the genetic code or (c) a nucleotide sequence hybridizing under stringent conditions to the sequence of (a) and/or (b).

26. A method for the in vitro enzymatic synthesis of deoxyribonucleosides comprising the steps of:

- condensing glyceraldehyde 3-phosphate (GAP) with acetaldehyde to deoxyribose 5-phosphate (dR5P),
- isomerizing deoxyribose 5-phosphate to deoxyribose 1-phosphate (dR1P) and
- reacting deoxyribose 1-phosphate and nucleobase, wherein a deoxyribonucleoside and inorganic phosphate are formed.

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27. The method of claim 26, wherein the reaction is carried out without isolating intermediate products.
28. The method of claim 26 or 27, wherein the glyceraldehyde 3-phosphate (GAP) is generated from fructose 1,6-diphosphate (FDP), dihydroxy-acetone (DHA) and/or glycerolphosphate (GP).
29. The method of claims 26 to 28, wherein before step (ii) excess acetaldehyde is removed.
30. The method of claims 26 to 29, wherein before step (ii) excess starting materials and/or by-products, particularly fructose 1,6-diphosphate and/or deoxyxylulose 1-phosphate (dX1P) are removed.
31. The method of claims 26 to 29, wherein the reaction is carried out in a manner that before step (ii) no substantial amounts of starting materials and/or by-products, particularly fructose 1,6-diphosphate and/or deoxyxylulose 1-phosphate are present.
32. A method for the in vitro enzymatic synthesis of deoxyribonucleosides comprising the steps of:
 - (i) phosphorylating deoxyribose to deoxyribose 5-phosphate,
 - (ii) isomerizing deoxyribose 5-phosphate to deoxyribose 1-phosphate and
 - (iii) reacting deoxyribose 1-phosphate and nucleobase, wherein a deoxyribonucleoside and inorganic phosphate are formed.
33. The method of claim 32, wherein the reaction is carried out without isolating intermediate products.

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34. The method of claims 26 to 33, wherein the inorganic phosphate is removed.
35. The method of any one of the previous claims comprising further reacting said deoxyribonucleoside.
36. The method of claim 35, wherein said further reacting comprises the synthesis of deoxyribonucleoside mono-, di- or triphosphates, of H-phosphonates or of phosphoramidites.
37. The use of an isolated nucleic acid molecule encoding a nucleoside 2-deoxyribosyl transferase (NdT, EC 2.4.2.6) for the preparation of an enzyme for an in vitro method for the enzymatic synthesis of deoxyribonucleosides, wherein a deoxyribonucleoside containing a first nucleobase is further reacted with a second nucleobase under formation of a deoxyribonucleoside containing the second nucleobase, wherein said nucleic acid molecule comprises (a) the nucleotide sequence shown in SEQ ID NO.13 or its complementary sequence, (b) a nucleotide sequence corresponding to the sequence of (a) in the scope of the degeneracy of the genetic code or (c) a nucleotide sequence hybridizing under stringent conditions to the sequence of (a) and/or (b).
38. The use of claim 37, wherein the second nucleobase is selected from cytidine and analogs thereof, e.g. 6-methyl purine, 2-amino-6-methylmercaptopurine, 6-dimethylaminopurine, 5-azacytidine, 2,6-dichloropurine, 6-chloroguanine, 6-chloropurine, 6-azathymine, 5-fluorouracil, ethyl-4-amino-5-imidazole carboxylate, imidazole-4-carboxamide and 1,2,4-triazole-3-carboxamide.
39. The use of claim 37 or 38, wherein the first nucleobase is selected from adenine, guanine, thymine, uracil and hypoxanthine.

AMENDMENT

40. The use of any one of claims 37-39, wherein the nucleic acid molecule is contained on a recombinant vector in operative linkage with an expression control sequence.
41. The use of any one of claims 37-40, wherein the nucleic acid is contained in a recombinant cell.
42. Use of an isolated polypeptide having NdT activity for the preparation of nucleosides according to claim 23.
43. Use of an isolated nucleic acid molecule encoding a deoxyribokinase (dRK, EC 2.7.1.5) for the preparation of an enzyme for an in vitro method for the enzymatic synthesis of deoxyribonucleosides comprising the step of phosphorylating deoxyribose to deoxyribose 5-phosphate, wherein said nucleic acid molecule comprises (a) the nucleotide sequence shown in SEQ ID NO.11 or its complementary sequence, (b) a nucleotide sequence corresponding to the sequence of (a) in the scope of the degeneracy of the genetic code or (c) a nucleotide sequence hybridizing under stringent conditions to the sequence of (a) and/or (b).
44. Use of an isolated polypeptide having dRK activity for an in vitro method for the enzymatic synthesis of deoxyribonucleosides comprising the step of phosphorylating deoxyribose to deoxyribose 5-phosphate.
45. Recombinant bacteria strains deposited at CNCM under accession numbers I-2186, I-2187, I-2188, I-2189, I-2190 and I-2191.

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(54) Title: ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEOSIDES

(57) Abstract: The present invention relates to a method for the in vitro enzymatic synthesis of deoxyribonucleosides and enzymes suitable for this method.

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Sakamoto, Hiroshi
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tgc cac tct tcc ggt acg gtc att ctg gat caa ctg ggc gaa gag cac 480
Cys His Ser Ser Gly Thr Val Ile Leu Asp Gln Leu Gly Glu Glu His
145 150 155 160

atg aaa acc ggc aag ccg att ttc tat acc tcc gct gac tcc gtg ttc 528
Met Lys Thr Gly Lys Pro Ile Phe Tyr Thr Ser Ala Asp Ser Val Phe
165 170 175

cag att gcc tgc cat gaa gaa act ttc ggt ctg gat aaa ctc tac gaa 576
Gln Ile Ala Cys His Glu Glu Thr Phe Gly Leu Asp Lys Leu Tyr Glu
180 185 190

ctg tgc gaa atc gcc cgt gaa gag ctg acc aac ggc ggc tac aat atc 624
Leu Cys Glu Ile Ala Arg Glu Glu Leu Thr Asn Gly Gly Tyr Asn Ile
195 200 205

ggt cgt gtt atc gct cgt ccg ttt atc ggc gac aaa gcc ggt aac ttc 672
Gly Arg Val Ile Ala Arg Pro Phe Ile Gly Asp Lys Ala Gly Asn Phe
210 215 220

cag cgt acc ggt aac cgt cac gac ctg gct gtt gag ccg cca gca ccg 720
Gln Arg Thr Gly Asn Arg His Asp Leu Ala Val Glu Pro Pro Ala Pro
225 230 235 240

acc gtg ctg cag aaa ctg gtt gat gaa aaa cac ggc cag gtg gtt tct 768
Thr Val Leu Gln Lys Leu Val Asp Glu Lys His Gly Gln Val Val Ser
245 250 255

gtc ggt aaa att gcg gac atc tac gcc aac tgc ggt atc acc aaa aaa 816
Val Gly Lys Ile Ala Asp Ile Tyr Ala Asn Cys Gly Ile Thr Lys Lys
260 265 270

gtg aaa gcg act ggc ctg gac gcg ctg ttt gac gcc acc atc aaa gag 864
Val Lys Ala Thr Gly Leu Asp Ala Leu Phe Asp Ala Thr Ile Lys Glu
275 280 285

atg aaa gaa gcg ggt gat aac acc atc gtc ttc acc aac ttc gtt gac 912
Met Lys Glu Ala Gly Asp Asn Thr Ile Val Phe Thr Asn Phe Val Asp
290 295 300

ttc gac tct tcc tgg ggc cac cgt cgc gac gtc gcc ggt tat gcc gcg 960
Phe Asp Ser Ser Trp Gly His Arg Arg Asp Val Ala Gly Tyr Ala Ala
305 310 315 320

ggt ctg gaa ctg ttc gac cgc cgt ctg ccg gag ctg atg tct ctg ctg 1008
Gly Leu Glu Leu Phe Asp Arg Arg Leu Pro Glu Leu Met Ser Leu Leu
325 330 335

cgc gat gac gac atc ctg atc ctc acc gct gac cac ggt tgc gat ccg 1056
Arg Asp Asp Asp Ile Leu Ile Leu Thr Ala Asp His Gly Cys Asp Pro
340 345 350

acc tgg acc ggt act gac cac acg cgt gaa cac att ccg gta ctg gta 1104
Thr Trp Thr Gly Thr Asp His Thr Arg Glu His Ile Pro Val Leu Val
355 360 365

tat ggc ccg aaa gta aaa ccg ggc tca ctg ggt cat cgt gaa acc ttc 1152
Tyr Gly Pro Lys Val Lys Pro Gly Ser Leu Gly His Arg Glu Thr Phe
370 375 380

gcg gat atc ggc cag act ctg gca aaa tat ttt ggt act tct gat atg 1200
Ala Asp Ile Gly Gln Thr Leu Ala Lys Tyr Phe Gly Thr Ser Asp Met
385 390 395 400

gaa tat ggc aaa gcc atg ttc tga 1224
Glu Tyr Gly Lys Ala Met Phe
405

<210> 6
<211> 407
<212> PRT
<213> Escherichia coli

<400> 6
Met Lys Arg Ala Phe Ile Met Val Leu Asp Ser Phe Gly Ile Gly Ala
1 5 10 15

Thr Glu Asp Ala Glu Arg Phe Gly Asp Val Gly Ala Asp Thr Leu Gly
20 25 30

His Ile Ala Glu Ala Cys Ala Lys Gly Glu Ala Asp Asn Gly Arg Lys
35 40 45

Gly Pro Leu Asn Leu Pro Asn Leu Thr Arg Leu Gly Leu Ala Lys Ala
50 55 60

His Glu Gly Ser Thr Gly Phe Ile Pro Ala Gly Met Asp Gly Asn Ala
65 70 75 80

Glu Val Ile Gly Ala Tyr Ala Trp Ala His Glu Met Ser Ser Gly Lys
85 90 95

Asp Thr Pro Ser Gly His Trp Glu Ile Ala Gly Val Pro Val Leu Phe
100 105 110

Glu Trp Gly Tyr Phe Ser Asp His Glu Asn Ser Phe Pro Gln Glu Leu
115 120 125

Leu Asp Lys Leu Val Glu Arg Ala Asn Leu Pro Gly Tyr Leu Gly Asn
130 135 140

Cys His Ser Ser Gly Thr Val Ile Leu Asp Gln Leu Gly Glu Glu His
145 150 155 160

Met Lys Thr Gly Lys Pro Ile Phe Tyr Thr Ser Ala Asp Ser Val Phe
165 170 175

Gln Ile Ala Cys His Glu Glu Thr Phe Gly Leu Asp Lys Leu Tyr Glu
180 185 190

Leu Cys Glu Ile Ala Arg Glu Glu Leu Thr Asn Gly Gly Tyr Asn Ile
195 200 205

Gly Arg Val Ile Ala Arg Pro Phe Ile Gly Asp Lys Ala Gly Asn Phe
210 215 220

Gln Arg Thr Gly Asn Arg His Asp Leu Ala Val Glu Pro Pro Ala Pro
225 230 235 240

Thr Val Leu Gln Lys Leu Val Asp Glu Lys His Gly Gln Val Val Ser
245 250 255

Val Gly Lys Ile Ala Asp Ile Tyr Ala Asn Cys Gly Ile Thr Lys Lys
260 265 270

Val Lys Ala Thr Gly Leu Asp Ala Leu Phe Asp Ala Thr Ile Lys Glu
275 280 285

Met Lys Glu Ala Gly Asp Asn Thr Ile Val Phe Thr Asn Phe Val Asp
290 295 300

Phe Asp Ser Ser Trp Gly His Arg Arg Asp Val Ala Gly Tyr Ala Ala
305 310 315 320

Gly Leu Glu Leu Phe Asp Arg Arg Leu Pro Glu Leu Met Ser Leu Leu
325 330 335

Arg Asp Asp Asp Ile Leu Ile Leu Thr Ala Asp His Gly Cys Asp Pro
340 345 350

Thr Trp Thr Gly Thr Asp His Thr Arg Glu His Ile Pro Val Leu Val
355 360 365

Tyr Gly Pro Lys Val Lys Pro Gly Ser Leu Gly His Arg Glu Thr Phe
370 375 380

Ala Asp Ile Gly Gln Thr Leu Ala Lys Tyr Phe Gly Thr Ser Asp Met
385 390 395 400

Glu Tyr Gly Lys Ala Met Phe
405

<210> 7

<211> 780

<212> DNA

<213> Escherichia coli

<220>

<221> CDS

<222> (1)..(777)

<400> 7

atg act gat ctg aaa gca agc agc ctg cgt gca ctg aaa ttg atg gac 48
Met Thr Asp Leu Lys Ala Ser Ser Leu Arg Ala Leu Lys Leu Met Asp
1 5 10 15

ctg aac acc ctg aat gac gac acc gac gag aaa gtg atc gcc ctg 96
Leu Asn Thr Leu Asn Asp Asp Asp Thr Asp Glu Lys Val Ile Ala Leu
20 25 30

tgt cat cag gcc aaa act ccg gtc ggc aat acc gcc gct atc tgt atc 144
Cys His Gln Ala Lys Thr Pro Val Gly Asn Thr Ala Ala Ile Cys Ile
35 40 45

tat cct cgc ttt atc ccg att gct cgc aaa act ctg aaa gag cag ggc 192
Tyr Pro Arg Phe Ile Pro Ile Ala Arg Lys Thr Leu Lys Glu Gln Gly
50 55 60

acc ccg gaa atc cgt atc gct acg gta acc aac ttc cca cac ggt aac 240
Thr Pro Glu Ile Arg Ile Ala Thr Val Thr Asn Phe Pro His Gly Asn
65 70 75 80

gac gac atc gac atc gcg ctg gca gaa acc cgt gcg gca atc gcc tac 288
Asp Asp Ile Asp Ile Ala Leu Ala Glu Thr Arg Ala Ala Ile Ala Tyr
85 90 95

ggt gct gat gaa gtt gac gtt gtg ttc ccg tac cgc gcg ctg atg gcg 336
Gly Ala Asp Glu Val Asp Val Val Phe Pro Tyr Arg Ala Leu Met Ala
100 105 110

ggt aac gag cag gtt ttt gac ctg gtg aaa gcc tgt aaa gag gct 384
Gly Asn Glu Gln Val Gly Phe Asp Leu Val Lys Ala Cys Lys Glu Ala
115 120 125

tgc gcg gca gcg aat gta ctg ctg aaa gtg atc atc gaa acc ggc gaa 432
Cys Ala Ala Ala Asn Val Leu Leu Lys Val Ile Ile Glu Thr Gly Glu
130 135 140

ctg aaa gac gaa gcg ctg atc cgt aaa gcg tct gaa atc tcc atc aaa 480
Leu Lys Asp Glu Ala Leu Ile Arg Lys Ala Ser Glu Ile Ser Ile Lys
145 150 155 160

gcg ggt gcg gac ttc atc aaa acc tct acc ggt aaa gtg gct gtg aac 528
Ala Gly Ala Asp Phe Ile Lys Thr Ser Thr Gly Lys Val Ala Val Asn
165 170 175

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gcg acg ccg gaa agc gcg cgc atc atg atg gaa gtg atc cgt gat atg 576
Ala Thr Pro Glu Ser Ala Arg Ile Met Met Glu Val Ile Arg Asp Met
180 185 190

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ggc gta gaa aaa acc gtt ggt ttc aaa ccg gcg ggc ggc gtg cgt act 624
Gly Val Glu Lys Thr Val Gly Phe Lys Pro Ala Gly Gly Val Arg Thr
195 200 205

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gcg gaa gat gcg cag aaa tat ctc gcc att gca gat gaa ctg ttc ggt 672
Ala Glu Asp Ala Gln Lys Tyr Leu Ala Ile Ala Asp Glu Leu Phe Gly
210 215 220

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gct gac tgg gca gat gcg cgt cac tac cgc ttt ggc gct tcc agc ctg 720
Ala Asp Trp Ala Asp Ala Arg His Tyr Arg Phe Gly Ala Ser Ser Leu
225           230           235           240

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ctg gca agc ctg ctg aaa gcg ctg ggt cac ggc gac ggt aag agc gcc 768
Leu Ala Ser Leu Leu Lys Ala Leu Gly His Gly Asp Gly Lys Ser Ala
245 250 255

agc agc tac taa 780
Ser Ser Tyr

<210> 8
<211> 259
<212> PRT
<213> Escherichia coli

<400> 8
Met Thr Asp Leu Lys Ala Ser Ser Leu Arg Ala Leu Lys Leu Met Asp
1 5 10 15

Leu Asn Thr Leu Asn Asp Asp Asp Thr Asp Glu Lys Val Ile Ala Leu
20 25 30

Cys His Gln Ala Lys Thr Pro Val Gly Asn Thr Ala Ala Ile Cys Ile
35 40 45

Tyr Pro Arg Phe Ile Pro Ile Ala Arg Lys Thr Leu Lys Glu Gln Gly
50 55 60

Thr Pro Glu Ile Arg Ile Ala Thr Val Thr Asn Phe Pro His Gly Asn
65 70 75 80

Asp Asp Ile Asp Ile Ala Leu Ala Glu Thr Arg Ala Ala Ile Ala Tyr
85 90 95

Gly Ala Asp Glu Val Asp Val Val Phe Pro Tyr Arg Ala Leu Met Ala
100 105 110

Gly Asn Glu Gln Val Gly Phe Asp Leu Val Lys Ala Cys Lys Glu Ala
115 120 125

Cys Ala Ala Ala Asn Val Leu Leu Lys Val Ile Ile Glu Thr Gly Glu
130 135 140

Leu Lys Asp Glu Ala Leu Ile Arg Lys Ala Ser Glu Ile Ser Ile Lys
145 150 155 160

Ala Gly Ala Asp Phe Ile Lys Thr Ser Thr Gly Lys Val Ala Val Asn
165 170 175

Ala Thr Pro Glu Ser Ala Arg Ile Met Met Glu Val Ile Arg Asp Met
180 185 190

Gly Val Glu Lys Thr Val Gly Phe Lys Pro Ala Gly Gly Val Arg Thr
195 200 205

Ala Glu Asp Ala Gln Lys Tyr Leu Ala Ile Ala Asp Glu Leu Phe Gly
210 215 220

Ala Asp Trp Ala Asp Ala Arg His Tyr Arg Phe Gly Ala Ser Ser Leu
225 230 235 240

Leu Ala Ser Leu Leu Lys Ala Leu Gly His Gly Asp Gly Lys Ser Ala
245 250 255

Ser Ser Tyr

<210> 9

<211> 1080

<212> DNA

<213> Escherichia coli

<220>

<221> CDS

<222> (1)..(1077)

<400> 9

atg tct aag att ttt gat ttc gta aaa cct ggc gta atc act ggt gat 48
Met Ser Lys Ile Phe Asp Phe Val Lys Pro Gly Val Ile Thr Gly Asp
1 5 10 15

gac gta cag aaa gtt ttc cag gta gca aaa gaa aac aac ttc gca ctg 96
Asp Val Gln Lys Val Phe Gln Val Ala Lys Glu Asn Asn Phe Ala Leu
20 25 30

cca gca gta aac tgc gtc ggt act gac tcc atc aac gcc gta ctg gaa 144
Pro Ala Val Asn Cys Val Gly Thr Asp Ser Ile Asn Ala Val Leu Glu

35 40 45

acc gct gct aaa gtt aaa gcg ccg gtt atc gtt cag ttc tcc aac ggt 192
Thr Ala Ala Lys Val Lys Ala Pro Val Ile Val Gln Phe Ser Asn Gly
50 55 60

ggg gct tcc ttt atc gct ggt aaa ggc gtg aaa tct gac gtt ccg cag 240
Gly Ala Ser Phe Ile Ala Gly Lys Gly Val Lys Ser Asp Val Pro Gln
65 70 75 80

ggg gct gct atc ctg ggc gcg atc tct ggt gcg cat cac gtt cac cag 288
Gly Ala Ala Ile Leu Gly Ala Ile Ser Gly Ala His His Val His Gln
85 90 95

atg gct gaa cat tat ggt gtt ccg gtt atc ctg cac act gac cac tgc 336
Met Ala Glu His Tyr Gly Val Pro Val Ile Leu His Thr Asp His Cys
100 105 110

gcg aag aaa ctg ctg ccg tgg atc gac ggt ctg ttg gac gcg ggt gaa 384
Ala Lys Lys Leu Leu Pro Trp Ile Asp Gly Leu Leu Asp Ala Gly Glu
115 120 125

aaa cac ttc gca gct acc ggt aag ccg ctg ttc tct cac atg atc 432
Lys His Phe Ala Ala Thr Gly Lys Pro Leu Phe Ser Ser His Met Ile
130 135 140

gac ctg tct gaa gaa tct ctg caa gag aac atc gaa atc tgc tct aaa 480
Asp Leu Ser Glu Glu Ser Leu Gln Glu Asn Ile Glu Ile Cys Ser Lys
145 150 155 160

tac ctg gag cgc atg tcc aaa atc ggc atg act ctg gaa atc gaa ctg 528
Tyr Leu Glu Arg Met Ser Lys Ile Gly Met Thr Leu Glu Ile Glu Leu
165 170 175

ggt tgc acc ggt ggt gaa gaa gac ggc gtg gac aac agc cac atg gac 576
Gly Cys Thr Gly Gly Glu Asp Gly Val Asp Asn Ser His Met Asp
180 185 190

gct tct gca ctg tac acc cag ccg gaa gac gtt gat tac gca tac acc 624
Ala Ser Ala Leu Tyr Thr Gln Pro Glu Asp Val Asp Tyr Ala Tyr Thr
195 200 205

gaa ctg agc aaa atc agc ccg cgt ttc acc atc gca gcg tcc ttc ggt 672
Glu Leu Ser Lys Ile Ser Pro Arg Phe Thr Ile Ala Ala Ser Phe Gly
210 215 220

aac gta cac ggt gtt tac aag ccg ggt aac gtg gtt ctg act ccg acc 720
Asn Val His Gly Val Tyr Lys Pro Gly Asn Val Val Leu Thr Pro Thr
225 230 235 240

atc ctg cgt gat tct cag gaa tat gtt tcc aag aaa cac aac ctg ccg 768
Ile Leu Arg Asp Ser Gln Glu Tyr Val Ser Lys Lys His Asn Leu Pro
245 250 255

cac aac agc ctg aac ttc gta ttc cac ggt ggt tcc ggt tct act gct 816
His Asn Ser Leu Asn Phe Val Phe His Gly Gly Ser Gly Ser Thr Ala
260 265 270

cag gaa atc aaa gac tcc gta agc tac ggc gta gta aaa atg aac atc 864
Gln Glu Ile Lys Asp Ser Val Ser Tyr Gly Val Val Lys Met Asn Ile
275 280 285

gat acc gat acc caa tgg gca acc tgg gaa ggc gtt ctg aac tac tac 912
Asp Thr Asp Thr Gln Trp Ala Thr Trp Glu Gly Val Leu Asn Tyr Tyr
290 295 300

aaa gcg aac gaa gct tat ctg cag ggt cag ctg ggt aac ccg aaa ggc 960
Lys Ala Asn Glu Ala Tyr Leu Gln Gly Gln Leu Gly Asn Pro Lys Gly
305 310 315 320

gaa gat cag ccg aac aag aaa tac tac gat ccg cgc gta tgg ctg cgt 1008
Glu Asp Gln Pro Asn Lys Lys Tyr Tyr Asp Pro Arg Val Trp Leu Arg
325 330 335

gcc ggt cag act tcg atg atc gct cgt ctg gag aaa gca ttc cag gaa 1056
Ala Gly Gln Thr Ser Met Ile Ala Arg Leu Glu Lys Ala Phe Gln Glu
340 345 350

ctg aac gcg atc gac gtt ctg taa 1080
Leu Asn Ala Ile Asp Val Leu
355

<210> 10
<211> 359
<212> PRT
<213> Escherichia coli

<400> 10
Met Ser Lys Ile Phe Asp Phe Val Lys Pro Gly Val Ile Thr Gly Asp
1 5 10 15

Asp Val Gln Lys Val Phe Gln Val Ala Lys Glu Asn Asn Phe Ala Leu
20 25 30

Pro Ala Val Asn Cys Val Gly Thr Asp Ser Ile Asn Ala Val Leu Glu
35 40 45

Thr Ala Ala Lys Val Lys Ala Pro Val Ile Val Gln Phe Ser Asn Gly
50 55 60

Gly Ala Ser Phe Ile Ala Gly Lys Gly Val Lys Ser Asp Val Pro Gln
65 70 75 80

Gly Ala Ala Ile Leu Gly Ala Ile Ser Gly Ala His His Val His Gln
85 90 95

Met Ala Glu His Tyr Gly Val Pro Val Ile Leu His Thr Asp His Cys
100 105 110

Ala Lys Lys Leu Leu Pro Trp Ile Asp Gly Leu Leu Asp Ala Gly Glu
115 120 125

Lys His Phe Ala Ala Thr Gly Lys Pro Leu Phe Ser Ser His Met Ile
130 135 140

Asp Leu Ser Glu Glu Ser Leu Gln Glu Asn Ile Glu Ile Cys Ser Lys
145 150 155 160

Tyr Leu Glu Arg Met Ser Lys Ile Gly Met Thr Leu Glu Ile Glu Leu
165 170 175

Gly Cys Thr Gly Gly Glu Asp Gly Val Asp Asn Ser His Met Asp
180 185 190

Ala Ser Ala Leu Tyr Thr Gln Pro Glu Asp Val Asp Tyr Ala Tyr Thr
195 200 205

Glu Leu Ser Lys Ile Ser Pro Arg Phe Thr Ile Ala Ala Ser Phe Gly
210 215 220

Asn Val His Gly Val Tyr Lys Pro Gly Asn Val Val Leu Thr Pro Thr
225 230 235 240

Ile Leu Arg Asp Ser Gln Glu Tyr Val Ser Lys Lys His Asn Leu Pro
245 250 255

His Asn Ser Leu Asn Phe Val Phe His Gly Gly Ser Gly Ser Thr Ala
260 265 270

Gln Glu Ile Lys Asp Ser Val Ser Tyr Gly Val Val Lys Met Asn Ile
275 280 285

Asp Thr Asp Thr Gln Trp Ala Thr Trp Glu Gly Val Leu Asn Tyr Tyr
290 295 300

Lys Ala Asn Glu Ala Tyr Leu Gln Gly Gln Leu Gly Asn Pro Lys Gly
305 310 315 320

Glu Asp Gln Pro Asn Lys Lys Tyr Tyr Asp Pro Arg Val Trp Leu Arg
325 330 335

Ala Gly Gln Thr Ser Met Ile Ala Arg Leu Glu Lys Ala Phe Gln Glu
340 345 350

Leu Asn Ala Ile Asp Val Leu
355

<210> 11

<211> 921

<212> DNA

<213> *Salmonella typhi*

<220>

<221> CDS

<222> (1)..(918)

<400> 11

atg gat atc gcg gtt att ggc tct aac atg gtg gac ctt atc acc tac 48

Met Asp Ile Ala Val Ile Gly Ser Asn Met Val Asp Leu Ile Thr Tyr

1

5

10

15

acc aac cag atg ccc aaa gaa ggg gaa act ctg gaa gcg ccg gcg ttt 96

Thr Asn Gln Met Pro Lys Glu Gly Thr Leu Glu Ala Pro Ala Phe

20

25

30

aaa atc ggc tgc ggc gga aaa ggg gcg aac cag qcc gtg gcg gcc gct 144

Lys Ile Gly Cys Gly Lys Gly Ala Asn Gln Ala Val Ala Ala Ala

35

40

45

aag ctc aat tca aaa gta ttg atg ttg acc aaa gtg ggc gac gat att 192

Lys Leu Asn Ser Lys Val Leu Met Leu Thr Lys Val Gly Asp Asp Ile

50

55

60

ttt gcc gac aac acc att cgt aat ctc gaa tcc tgg ggg atc aat acg 240

Phe Ala Asp Asn Thr Ile Arg Asn Leu Glu Ser Trp Gly Ile Asn Thr

65

70

75

80

acg tat gta gaa aaa gta ccg tgt acc agc agc ggc gta gcg ccg att 288

Thr Tyr Val Glu Lys Val Pro Cys Thr Ser Ser Gly Val Ala Pro Ile

85

90

95

ttc gtc aac gcc aac tcc agc aac agc att ctg atc atc aaa ggc gct 336
Phe Val Asn Ala Asn Ser Ser Asn Ser Ile Leu Ile Ile Lys Gly Ala
100 105 110

aac aag ttt ctc tcg ccg gaa gat atc gat cgc gcg gaa gat tta 384
Asn Lys Phe Leu Ser Pro Glu Asp Ile Asp Arg Ala Ala Glu Asp Leu
115 120 125

aaa aaa tgc cag ctt att gtt ctg caa ctg gaa gtt cag ctt gaa acg 432
Lys Lys Cys Gln Leu Ile Val Leu Gln Leu Glu Val Gln Leu Glu Thr
130 135 140

gtt tat cac gca ata gaa ttt ggc aag aaa cac ggg att gaa gtg tta 480
Val Tyr His Ala Ile Glu Phe Gly Lys Lys His Gly Ile Glu Val Leu
145 150 155 160

tta aac cct gcg cca gca tta cgg gaa tta gat atg tct tat gcc tgt 528
Leu Asn Pro Ala Pro Ala Leu Arg Glu Leu Asp Met Ser Tyr Ala Cys
165 170 175

aaa tgc gat ttc ttt gta cct aat gaa acc gag ctg gaa ata tta acc 576
Lys Cys Asp Phe Phe Val Pro Asn Glu Thr Glu Leu Glu Ile Leu Thr
180 185 190

ggg atg cca gtg gat acc tat gac cat att cgc gca gcg gca cgt tcg 624
Gly Met Pro Val Asp Thr Tyr Asp His Ile Arg Ala Ala Ala Arg Ser
195 200 205

ctg gta gat aaa ggg ctg aac aat att att gtc acc atg ggc gag aaa 672
Leu Val Asp Lys Gly Leu Asn Asn Ile Ile Val Thr Met Gly Glu Lys
210 215 220

ggc gcg ctg tgg atg acg cgt gac cag gaa gtc cat gtt ccg gcg ttt 720
Gly Ala Leu Trp Met Thr Arg Asp Gln Glu Val His Val Pro Ala Phe
225 230 235 240

aga gtg aac gct gtt gat acc agc ggc gcg ggc gat gcc ttt atc ggc 768
Arg Val Asn Ala Val Asp Thr Ser Gly Ala Gly Asp Ala Phe Ile Gly
245 250 255

tgt ttc gcg cat tac tac gtc cag agc ggg gat gtg gaa gcc gcc atg 816
Cys Phe Ala His Tyr Tyr Val Gln Ser Gly Asp Val Glu Ala Ala Met
260 265 270

aaa aaa gcc gtc ctc ttt gcc gct ttc agc gtc acc ggg aaa ggc acc 864
Lys Lys Ala Val Leu Phe Ala Ala Phe Ser Val Thr Gly Lys Gly Thr
275 280 285

caa tcc tct tat cca agc att gag caa ttt aat gag tat ctt tcg ttg 912
Gln Ser Ser Tyr Pro Ser Ile Glu Gln Phe Asn Glu Tyr Leu Ser Leu
290 295 300

aac gaa taa 921
Asn Glu
305

<210> 12
<211> 306
<212> PRT
<213> *Salmonella typhi*

<400> 12
Met Asp Ile Ala Val Ile Gly Ser Asn Met Val Asp Leu Ile Thr Tyr
1 5 10 15

Thr Asn Gln Met Pro Lys Glu Gly Glu Thr Leu Glu Ala Pro Ala Phe
20 25 30

Lys Ile Gly Cys Gly Gly Lys Gly Ala Asn Gln Ala Val Ala Ala Ala
35 40 45

Lys Leu Asn Ser Lys Val Leu Met Leu Thr Lys Val Gly Asp Asp Ile
50 55 60

Phe Ala Asp Asn Thr Ile Arg Asn Leu Glu Ser Trp Gly Ile Asn Thr
65 70 75 80

Thr Tyr Val Glu Lys Val Pro Cys Thr Ser Ser Gly Val Ala Pro Ile
85 90 95

Phe Val Asn Ala Asn Ser Ser Asn Ser Ile Leu Ile Ile Lys Gly Ala
100 105 110

Asn Lys Phe Leu Ser Pro Glu Asp Ile Asp Arg Ala Ala Glu Asp Leu
115 120 125

Lys Lys Cys Gln Leu Ile Val Leu Gln Leu Glu Val Gln Leu Glu Thr
130 135 140

Val Tyr His Ala Ile Glu Phe Gly Lys Lys His Gly Ile Glu Val Leu
145 150 155 160

Leu Asn Pro Ala Pro Ala Leu Arg Glu Leu Asp Met Ser Tyr Ala Cys
165 170 175

Lys Cys Asp Phe Phe Val Pro Asn Glu Thr Glu Leu Glu Ile Leu Thr
180 185 190

Gly Met Pro Val Asp Thr Tyr Asp His Ile Arg Ala Ala Ala Arg Ser
195 200 205

Leu Val Asp Lys Gly Leu Asn Asn Ile Ile Val Thr Met Gly Glu Lys
210 215 220

Gly Ala Leu Trp Met Thr Arg Asp Gln Glu Val His Val Pro Ala Phe
225 230 235 240

Arg Val Asn Ala Val Asp Thr Ser Gly Ala Gly Asp Ala Phe Ile Gly
245 250 255

Cys Phe Ala His Tyr Tyr Val Gln Ser Gly Asp Val Glu Ala Ala Met
260 265 270

Lys Lys Ala Val Leu Phe Ala Ala Phe Ser Val Thr Gly Lys Gly Thr
275 280 285

Gln Ser Ser Tyr Pro Ser Ile Glu Gln Phe Asn Glu Tyr Leu Ser Leu
290 295 300

Asn Glu
305

<210> 13
<211> 483
<212> DNA
<213> Lactobacillus leichmannii

<220>
<221> CDS

25135563v1.w

<222> (10) .. (480)

<400> 13

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 Met Pro Lys Lys Thr Ile Tyr Phe Gly Ala Gly Trp Phe Thr
 1 5 10

gac cgc caa aac aaa gcc tac aag gaa gcc atg gaa gcc ctc aag gaa 99
 Asp Arg Gln Asn Lys Ala Tyr Lys Glu Ala Met Glu Ala Leu Lys Glu
 15 20 25 30

aac cca acg att gac ctg gaa aac agc tac gtt ccc ctg gac aac cag 147
 Asn Pro Thr Ile Asp Leu Glu Asn Ser Tyr Val Pro Leu Asp Asn Gln
 35 40 45

tac aag ggt atc cgg gtt gat gaa cac ccg gaa tac ctg cat gac aag 195
 Tyr Lys Gly Ile Arg Val Asp Glu His Pro Glu Tyr Leu His Asp Lys
 50 55 60

gtt tgg gct acg gcc acc tac aac aac gac ttg aac ggg atc aag acc 243
 Val Trp Ala Thr Ala Thr Tyr Asn Asn Asp Leu Asn Gly Ile Lys Thr
 65 70 75

aac gac atc atg ctg ggt gtc tac atc cct gac gaa gaa gac gtc ggc 291
 Asn Asp Ile Met Leu Gly Val Tyr Ile Pro Asp Glu Glu Asp Val Gly
 80 85 90

ctg ggc atg gaa ctg ggt tac gcc ttg agc caa ggc aag tac gtc ctt 339
 Leu Gly Met Glu Leu Gly Tyr Ala Leu Ser Gln Gly Lys Tyr Val Leu
 95 100 105 110

ttg gtc atc ccg gac gaa gac tac ggc aag ccg atc aac ctc atg agc 387
 Leu Val Ile Pro Asp Glu Asp Tyr Gly Lys Pro Ile Asn Leu Met Ser

115

120

125

tgg ggc gtc agc gac aac gtg atc aag atg agc cag ctg aag gac ttc 435
Trp Gly Val Ser Asp Asn Val Ile Lys Met Ser Gln Leu Lys Asp Phe
130 135 140

aac ttc aac aag ccg cgc ttc gac ttc tac gaa ggt gcc gta tac taa 483
Asn Phe Asn Lys Pro Arg Phe Asp Phe Tyr Glu Gly Ala Val Tyr
145 150 155

<210> 14

<211> 157

<212> PRT

<213> Lactobacillus leichmannii

<400> 14

Met Pro Lys Lys Thr Ile Tyr Phe Gly Ala Gly Trp Phe Thr Asp Arg
1 5 10 15

Gln Asn Lys Ala Tyr Lys Glu Ala Met Glu Ala Leu Lys Glu Asn Pro
20 25 30

Thr Ile Asp Leu Glu Asn Ser Tyr Val Pro Leu Asp Asn Gln Tyr Lys
35 40 45

Gly Ile Arg Val Asp Glu His Pro Glu Tyr Leu His Asp Lys Val Trp
50 55 60

Ala Thr Ala Thr Tyr Asn Asn Asp Leu Asn Gly Ile Lys Thr Asn Asp
65 70 75 80

Ile Met Leu Gly Val Tyr Ile Pro Asp Glu Glu Asp Val Gly Leu Gly
85 90 95

Met Glu Leu Gly Tyr Ala Leu Ser Gln Gly Lys Tyr Val Leu Leu Val
100 105 110

Ile Pro Asp Glu Asp Tyr Gly Lys Pro Ile Asn Leu Met Ser Trp Gly
115 120 125

Val Ser Asp Asn Val Ile Lys Met Ser Gln Leu Lys Asp Phe Asn Phe
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Fig. 1

dR5P-Synthesis / TS_09_02_00 #4

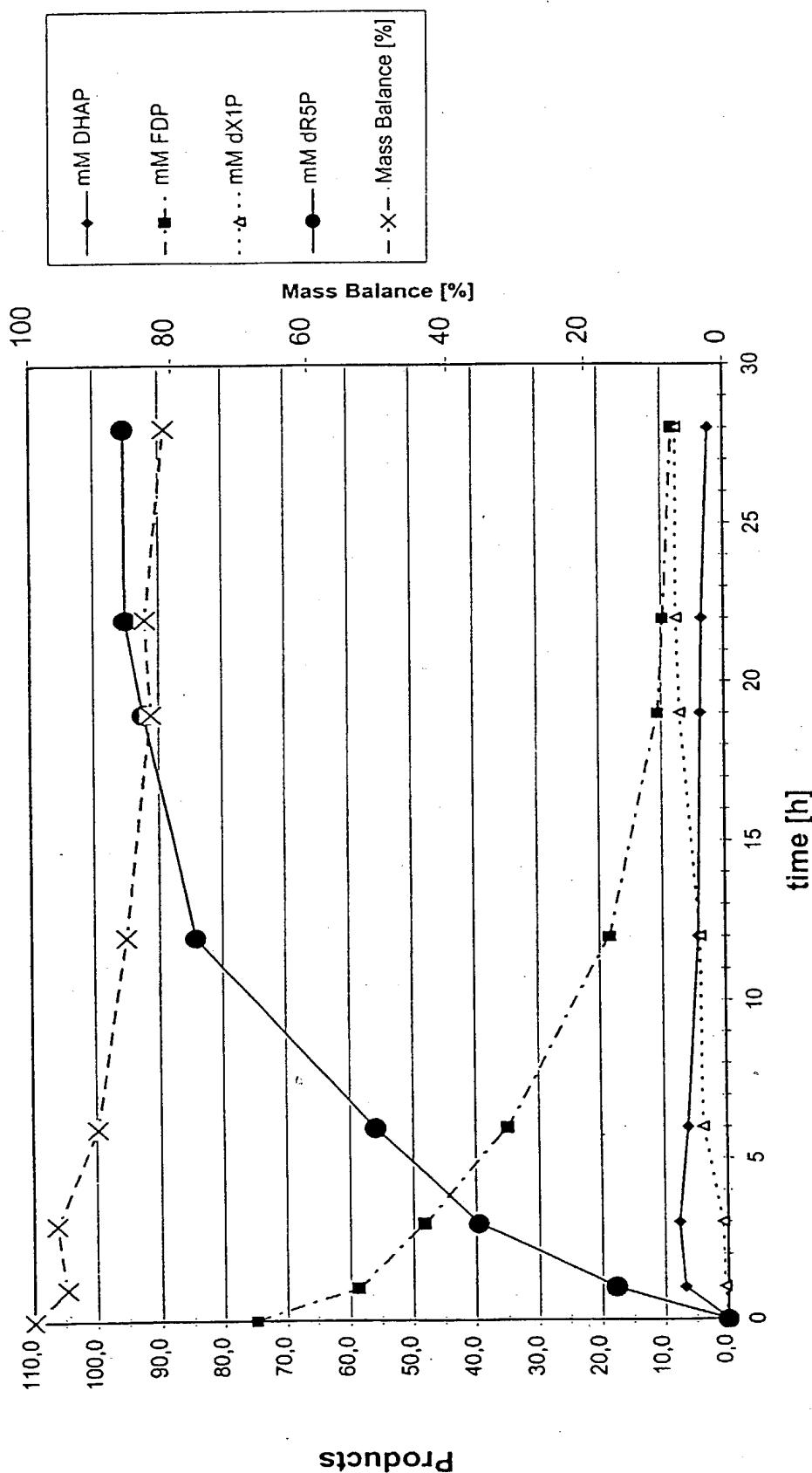


Fig. 2

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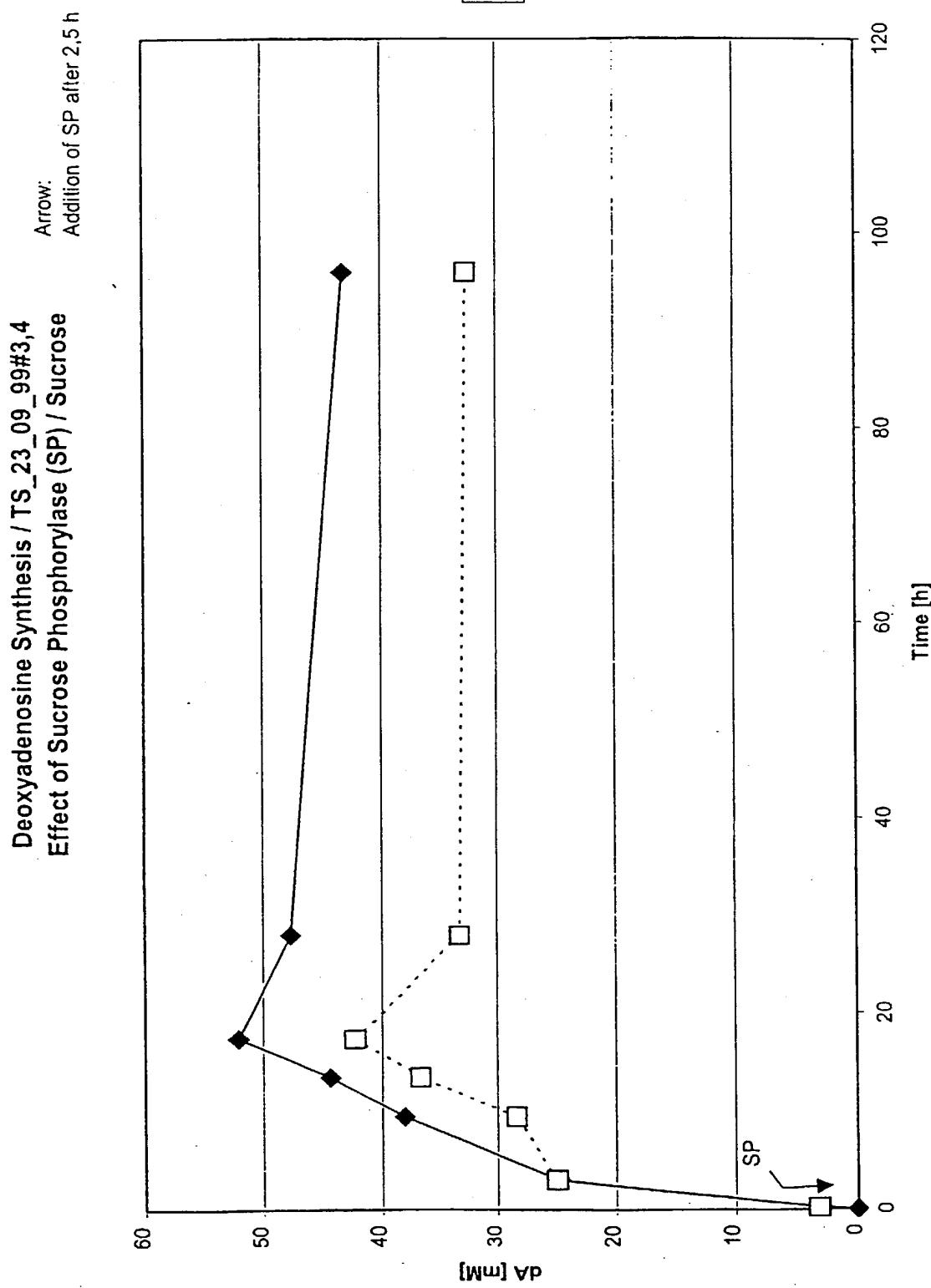
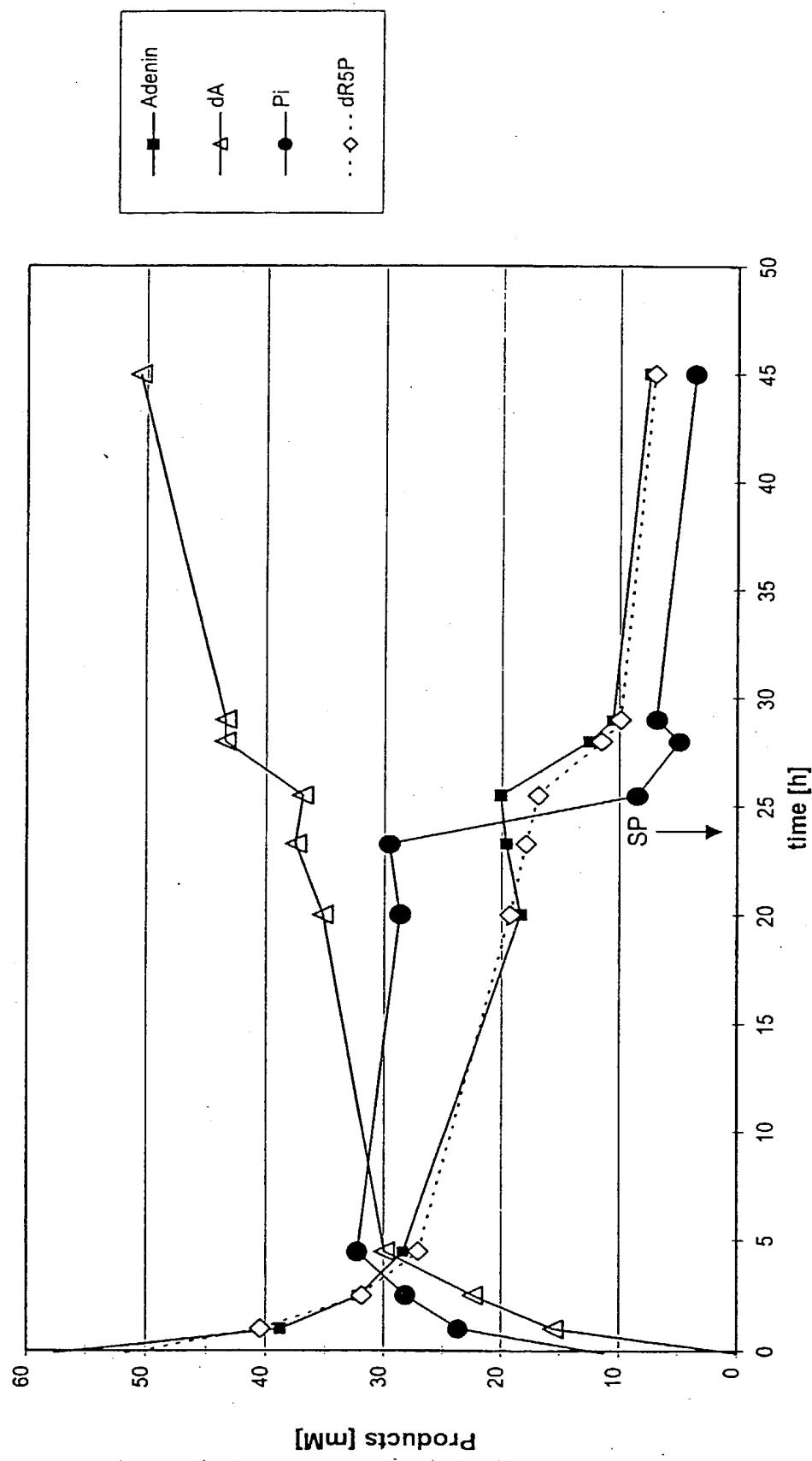


Fig. 3

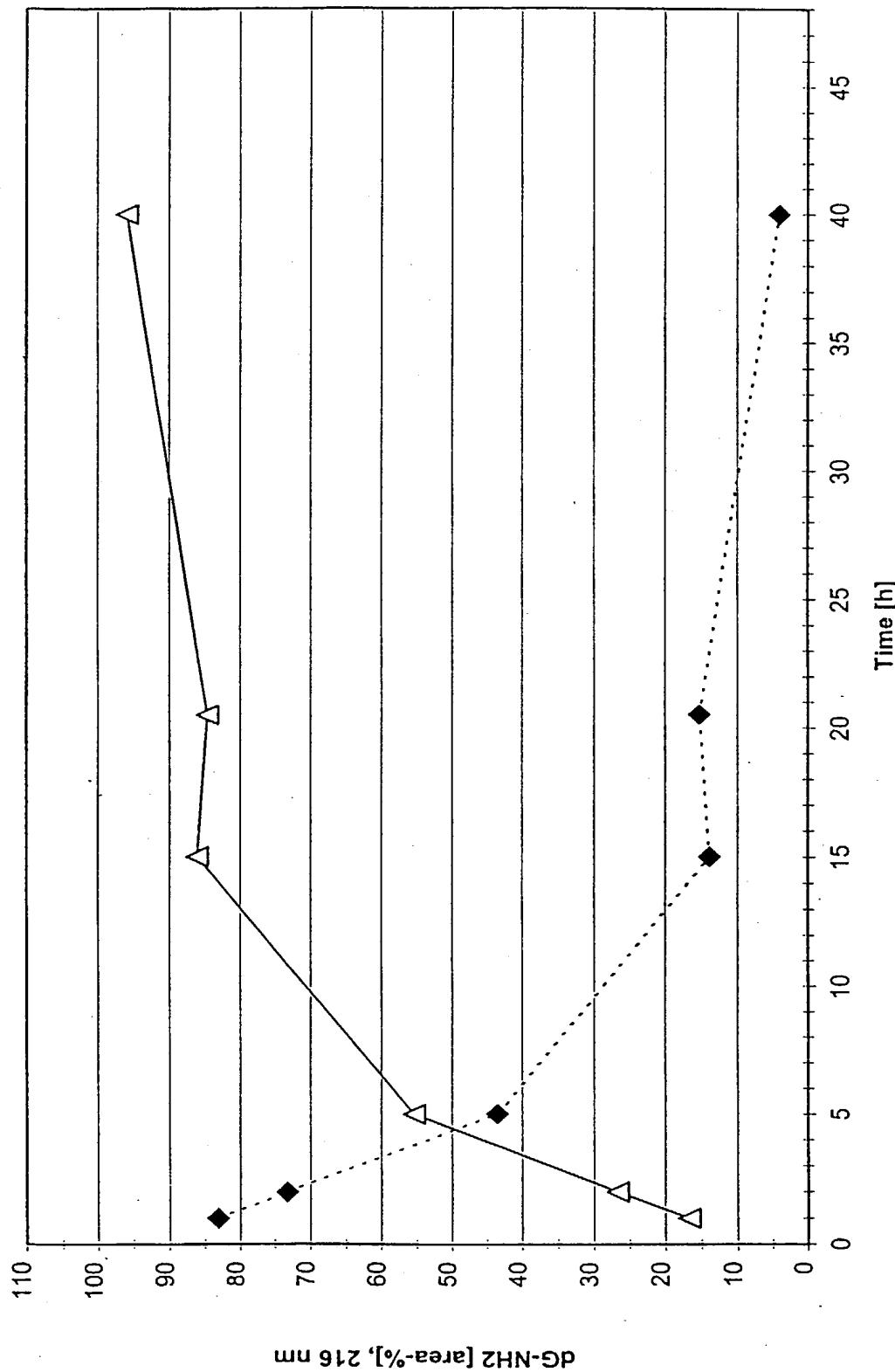
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Deoxyadenosine Synthesis / TS_08_12_99#1
 Effect of Sucrose Phosphorylase (SP)/Sucrose

Arrow:
 Addition of SP

4/4

Fig. 4

dG-NH₂ Synthesis / TS_dG-NH₂_29_06_00#6

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My resident, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled "Enzymatic Synthesis of Deoxyribonucleosides", the specification of which

() is attached hereto. International
(x) was filed on August 18, 2000 as Application Serial No. PCI/EP00/08088

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

Foreign Priority Applications

I hereby claim foreign priority benefits under Title 35, United States Code 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Priority Claimed

99 116 425.2 Europe 20/August/1999 Yes No
(Number) (Country) (Day/Month/Year Filed)

Yes () No ()
(Number) (Country) (Day/Month/Year Filed)

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

(Application Number)

(Filing Date)

U.S. Priority Applications

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Applic. Serial No.)	(Filing Date)	(Status-patented/pending/abandoned)
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(Applic. Serial No.)	(Filing Date)	(Status-patented/pending/abandoned)
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Power of Attorney

I hereby appoint the following attorneys and patent agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: Peter F. Felfe, Reg. No. 20,297; John E. Lynch, Reg. No. 20,940; Norman D. Hanson, Reg. No. 30,946; John A. Bauer, Reg. No. 32,554; Mary Anne Schofield, Reg. No. 36,669; James Zubok, Reg. No. 38,671; James R. Crawford, Reg. No. 39,155, Andrew Im, Reg. No. 40,657 and David Rubin, Reg. No. 40,314; my attorneys with full power of substitution and revocation.

CUSTOMER no. 24972

Address all telephone calls to _____ at (212) 318-3000.

Address all correspondence to: FULBRIGHT & JAWORSKI L.L.P.
666 Fifth Avenue
New York, New York 10103

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00
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Full Name/Sole or First Inventor

Wolfgang Tischer

04/08/02

Signature

Date

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(2) Hans-Georg Ihlenfeldt

Full Name/Second Inventor

Hans GeorgIhlenfeldt

Signature

Date

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82393 Iffeldorf, GermanyCitizenship: German

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Full Name/Third Inventor

Signature

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Date

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Citizenship: French

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(4) Hiroshi Sakamoto

Full Name/Fourth Inventor

Signature

April 23, 2002

Date

Residence: F-92190 Meudon, France

FRL

Post Office Address: 3, sentier des Jardies
F-92190 Meudon, France

Citizenship: Japan

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(5) Elisabeth Pistotnik

Full Name/Fifth Inventor

E. Pistotnik

June 11, 2002

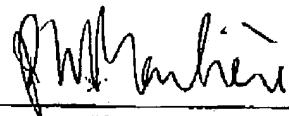
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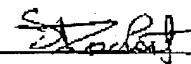
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U⁰⁰(6) Philippe MarlièreFull Name / sixth inventor

Signature

September 10, 2002

Date

Residence: F-91450 Etiolles, FrancePost Office Address: 2, allée Saint Martin
91450 Etiolles, FranceCitizenship: French(7) Sylvie PocheFull Name / Seventh inventor

May 23, 2002

Residence: F-75012 Paris, FrancePost Office Address: 5, rue Gossec
75012 Paris, FranceCitizenship: French

Full Name /

Signature

Date

Residence:



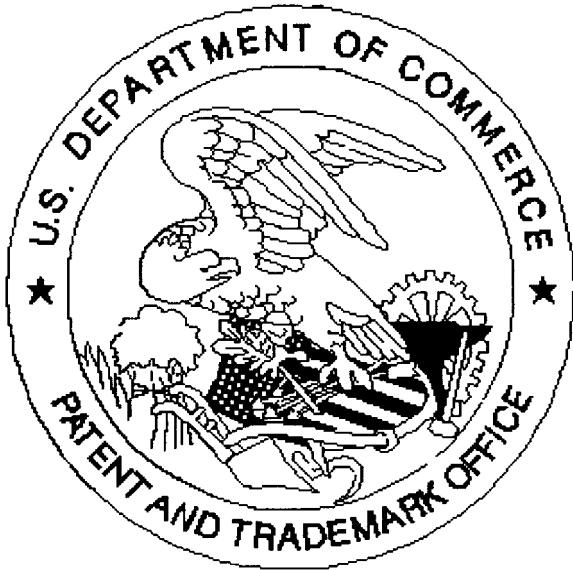
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Office of Initial Patent Examination -- Scanning Division



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